

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

<i>In re</i> Application of:)	Group Art Unit: 1633
)	
William Beschorner <i>et al.</i>)	Examiner: SAJJADI
)	
Serial No.: 10/527,587)	Atty. Docket No. 000241.00003
)	
Filed: February 21, 2006)	Confirmation No. 1006

For: **GROWTH OF FOREIGN CELLS IN FETAL ANIMALS FACILITATED BY
CONDITIONAL AND SELECTIVE DESTRUCTION OF NATIVE HOST CELLS**

DECLARATION OF DR. WILLIAM E. BESCHORNER UNDER 37 C.F.R. § 1.131

U.S. Patent and Trademark Office
Randolph Building
401 Dulany Street
Alexandria, VA 22314

I, William E. Beschorner, declare as follows:

1. I am named as an inventor of the subject matter claimed in Serial No. 10/527,587. Carlos E. Sosa and Scott C. Thompson are non-signing inventors of the subject matter claimed in Serial No. 10/527,587. I am an applicant under 37 C.F.R § 1.47(a) by petition granted on October 27, 2006. I am President and Chief Scientific Officer of Ximerex, Inc., to which this application is assigned. My curriculum vitae is attached as **Exhibit 1**.

2. All work described in this declaration was performed in the United States.

3. Before November 2, 1999, we conceived of a method of engrafting foreign replacement cells within a fetal non-human mammal. The method involved steps of selectively destroying native cells in a tissue of a fetal non-human mammal host without substantially

reducing the number of maternal cells of the same tissue and then implanting foreign replacement cells in the tissue of the fetal non-human mammal host. Using this method, the foreign replacement cells would replace destroyed cells of the tissue.

4. **Exhibit 2** is a copy of a grant application which we submitted to the National Institute of Standards and Technology Advanced Technology Program (ATP) for funding in March 1999 and which describes this method. For example, paragraphs 6 and 7 on page 1 describe culturing human hepatocytes in pre-immune fetal pigs and providing a growth advantage to the human cells by selectively targeting pig cells for destruction by selectively expressing suicide genes in the pig cells. Paragraph 7 explains that the suicide genes encode proteins that convert non-toxic prodrugs into toxic drugs and include thymidine kinase and cytosine deaminase.

5. The following paragraphs demonstrate our efforts to reduce this method to practice, which involved the following stages:

- demonstration of hepatocellular chimerism in wild-type pigs;
- design of constructs used to prepare transgenic pigs;
- improved techniques for hepatocyte administration;
- development of transfection techniques for making transgenic pigs;
- development of mouse transgenic model;
- development of improved constructs for optimized expression;
- creation of transgenic animals containing a suicide gene;

- creation of transgenic pigs containing the mutated thymidine kinase suicide gene; and
- preparation and filing of a provisional patent application which describes the method and how to carry it out.

6. The Exhibits to this Declaration are true copies of the original documents except for the addition of text to identify the documents and brackets to assist in locating the portions of the Exhibits which are discussed below.

Demonstration of Hepatocellular Chimerism in Wild-Type Pigs

7. Between March 1999 and June 1999, we infused human hepatocytes into wild-type fetal pigs and determined that the growth was limited due to competition from native pig hepatocytes.

8. **Exhibit 3** is a copy of laboratory notebook page 37, dated March 17, 1999, which describes preparation of human hepatocytes.

9. **Exhibit 4** is a copy of laboratory notebook page 189, dated March 17, 1999, which describes injection of human hepatocytes into the livers of fetal pigs of Sow #75.

10. **Exhibit 5** is a copy of laboratory notebook page 201. The entry dated May 22, 1999 notes that Sow #75 delivered three live piglets as well as five stillborn and one mummified fetus.

11. **Exhibit 6** is a copy of notebook page 202, dated May 26, 1999, which documents euthanasia of the three live piglets and assessment of their livers.

12. **Exhibit 7** contains copies of laboratory notebook page 204, dated June 9, 1999, and page 205, dated June 10, 1999, which report the results of histological examination of the piglets' livers. See paragraphs 1-3 on notebook page 204, the table on notebook page 205, and the histology photographs on pages 206 and 207 dated June 13, 1999.

13. **Exhibit 8** is a copy of laboratory notebook page 210, dated June 13, 1999, which reports that PCR experiments confirmed the presence of hepatocellular chimerism in the spleens of piglets delivered from Sow #75.

14. **Exhibit 9** contains copies of laboratory notebook pages 70 to 72, dated May 24, 26 and May 28, 1999, which record the results of histological and PCR analysis of piglet spleens for the presence of human hepatocytes.

15. **Exhibit 10** is a portion of a Small Business Innovation Research grant application submitted to the National Institutes of Health in August 1999. The third paragraph on page 3 of **Exhibit 10** (page 18 of the grant application) reports that measured levels of the human proteins alpha-1 antitrypsin (AAT) and serum amyloid A (SAA) indicated the presence of human hepatocytes in the three live piglets.

16. **Exhibit 11** contains copies of pages 4-11 and 4-12 of an ATP grant application dated March 7, 2000 and provides data which demonstrates that human hepatocytes proliferated in the spleens of piglets delivered from Sow #75. See the bracketed section of the table on page 4-12.

Design of Constructs Used to Prepare Transgenic Pigs

17. Between March 1999 and April 2000, we designed and made constructs encoding suicide genes such as thymidine kinase and marker proteins such as green fluorescent protein (GFP) to use in making the transgenic pigs.

18. **Exhibit 12** is a copy of a laboratory notebook page dated January 2000, which records diagrams of constructs. See the bracketed portion at the center of the page.

19. **Exhibit 13** is a copy of page 24 of an ATP grant application dated April 13, 1999. The bracketed paragraph describes the development of transgene constructs and vectors.

20. **Exhibit 14** is a copy of page 4-17 of the ATP grant application dated March 7, 2000. The bracketed paragraphs discuss several strategies for using our suicide gene approach.

Improved Techniques for Hepatocyte Administration

21. Between June 1999 and November 1999 we improved our methods of preparing human hepatocytes and developed more effective techniques to improve pig viability after infusion of human hepatocytes into fetal pigs.

22. **Exhibit 15** is a copy of laboratory notebook page 215, dated June 20, 1999, which describes the results of pig viability studies.

23. Between July 1999 and October 1999, we used our improved techniques to implant hepatocytes into fetal pigs. The improved techniques gave higher SAA levels compared to previous approaches. **Exhibit 16** contains copies of laboratory notebook pages which describe experiments which resulted in improved piglet survival. Notebook page 235, dated July 21,

1999, documents injection of Sow #316 with hepatocytes. Notebook page 236, dated July 22, 1999, documents injection of Sows #107 and #173 with hepatocytes. Notebook page 246, with entries from August 27, 1999 to September 8, 1999, documents gestation of the pig fetuses. Notebook page 251, with entries dated September 29, 1999 to October 5, 1999, notes on October 4, 1999 that the piglets were doing well and gaining weight. Notebook page 253, dated October 11, 1999, also notes that pigs #3, 4, and 6 were still doing well.

24. **Exhibit 17** contains copies of laboratory notebook pages which document validation of the ELISA assay to measure SAA levels and application of the assay to piglets from sows. These experiments in **Exhibit 17** were carried out between August 2, 1999 and January 12, 2000, for example on August 2 (notebook page 41; **Exhibit 17**, page 1), August 4 (**Exhibit 17**, page 2), August 6 (**Exhibit 17**, page 3), August 10 (**Exhibit 17**, page 4), September 10 (**Exhibit 17**, page 5), September 15 and 17 (**Exhibit 17**, page 6), September 22 and 24 (**Exhibit 17**, page 7), October 15 (**Exhibit 17**, page 8), October 15, 18, and 20 (**Exhibit 17**, page 9), October 27 and 29 (**Exhibit 17**, page 10), and November 11 and 19 and January 12 (**Exhibit 17**, page 11).

25. **Exhibit 18** is a copy of page 4-12 of our ATP grant application dated March 7, 2000, which summarizes the experiments described in **Exhibit 17** for piglets 316-1, 316-2, 316-3, 361-1, and 361-2 (bracketed section of the table on page 4-12). SAA levels were higher for those piglets than piglets 75-1 to 75-6, which were made using the older protocol.

26. Between October 1999 and January 2000, we monitored survival of the human hepatocytes in pig livers and determined that while the hepatocytes were not rejected rapidly, they gradually died over time. **Exhibit 19** summarizes the results of experiments showing

decreased survival as monitored by decline in SAA levels. Data from piglet 316-6, for example, illustrates the decline in SAA production from 250 ng/ml to zero between October 4, 1999 and January 12, 2000.

Development of Transfection Techniques for Making Transgenic Pigs

27. Between January 2000 and June 2000, we developed transfection procedures using retroviral vector technology and applied it to pigs. We used retroviral vectors to transfect genes into porcine PK-15 cells to establish that nuclear transfer technology would work in pigs.

28. **Exhibit 20** contains pages from a laboratory notebook that describe experiments optimizing PCR conditions to develop vectors. The bracketed section on page 79, dated April 28, 2000, shows that we obtained PCR product using an extension temperature of 61°C. These experiments in **Exhibit 20** were carried out between April 4, 2000 and June 2, 2000, for example on April 4 (**Exhibit 20**, page 1), April 5 (**Exhibit 20**, page 2), April 6 (**Exhibit 20**, page 3), April 7, 10 (**Exhibit 20**, page 4), April 25, 26 (**Exhibit 20**, page 5), April 26, 27 (**Exhibit 20**, page 6), April 28 (**Exhibit 20**, page 7), May 1 (**Exhibit 20**, page 8), Between May 1 and May 3 (**Exhibit 20**, page 9), May 3, 4 (**Exhibit 20**, page 10), May 5 (**Exhibit 20**, page 11), May 8, 9 (**Exhibit 20**, page 12), May 10 (**Exhibit 20**, page 13), May 11 (**Exhibit 20**, page 14), May 12, 13 (**Exhibit 20**, page 15), Between May 13 and May 24 (**Exhibit 20**, page 16), May 24, 25 (**Exhibit 20**, page 17), May 29, 30 (**Exhibit 20**, page 18), May 31 (**Exhibit 20**, page 19), June 5, 6 (**Exhibit 20**, page 20), June 7 (**Exhibit 20**, page 22), June 8, 9, 12, 13 (**Exhibit 20**, page 23),

June 14, 15 (**Exhibit 20**, page 24), June 19 (**Exhibit 20**, page 25), and June 20, 22 (**Exhibit 20**, page 26).

29. **Exhibit 21** contains pages of our ATP grant application dated March 7, 2000. Page 4-2 of the application describes administering pro-drug to sows. Page 4-12 of the application (bracketed paragraphs) reports that we achieved continued expression of the GFP marker gene over a three week period in PK-15 cells transfected with the retroviral vector.

30. Between July 2000 and January 2001, we developed multiple transgene constructs and identified promoters that allow both universal and liver-specific expression of proteins in pigs. **Exhibit 22** contains notebook pages describing our design for constructs encoding cytosine deaminase and GFP. These experiments in **Exhibit 22** were carried out between November 2000 4, 2000 and January 2001, for example on November 9, 13 (**Exhibit 22**, page 1), November 14 (**Exhibit 22**, page 2), November 13 and 14, December 6 and 20 (**Exhibit 22**, page 3), December 6 (**Exhibit 22**, page 4), November 20, 30 (**Exhibit 22**, page 5), November 13, 20 (**Exhibit 22**, page 6), November 14, 17 (**Exhibit 22**, page 7), November 15, 16, 17, 20, 21 (**Exhibit 22**, page 8), November 16, 28, 29, 30 (**Exhibit 22**, page 9), and November 28, January 10 (**Exhibit 22**, page 10).

31. **Exhibit 23** contains pages 3-5 of a quarterly progress report for our ATP grant for November/December 2000. The report identifies promoters for universal and specific expression and discusses additional constructs. Bracketed paragraph 4 on page 4 describes four constructs.

32. **Exhibit 24** is a diagram from a presentation given on July 13, 2000 and illustrates a transgene construct designed to demonstrate expression of suicide genes and green fluorescent protein expression in liver and control tissues.

Development of Mouse Transgenic Model

33. Between June 2000 and December 2001, we developed a transgenic mouse hybrid liver model. We also designed additional constructs to achieve localized expression of the suicide gene driven by the albumin promoter. **Exhibit 25** is a copy of a letter I wrote to the co-chairs of the Institutional Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center, dated July 5, 2000, requesting expedited review of our experimental protocols for developing transgenic mice and pigs. The IACUC's approval of our protocol was required before these studies could begin.

34. **Exhibit 26** is a copy of a letter I wrote to the director of Transgenic Core Laboratory, dated November 15, 2000, requesting assistance in developing transgenic mice.

35. **Exhibit 27** is a copy of page 6 of a November-December 2000 quarterly report for our ATP grant which describes our progress towards obtaining a functional mouse transgenic support facility.

36. **Exhibit 28** is a copy of a letter I wrote to the director of Transgenic Core Laboratory, dated March 20, 2001, finalizing the contract terms for preparing the transgenic mice.

37. **Exhibit 29** contains copies of two pages of a laboratory notebook dated September 18 and 19, 2001, respectively which describe vectors for use in creating the transgenic mice.

38. **Exhibit 30** is a copy of page 7 of a quarterly report for our ATP grant, covering April 2001 to June 2001, which reports that an outbreak of hepatitis forced a postponement of the production of transgenic mice (item 5).

39. **Exhibit 31** is a copy of page 7 of a quarterly report for our ATP grant, covering July 2001 to October 2001 explaining that candidate founder mice had been bred and were being screened for the transgene.

40. **Exhibit 32** contains copies of pages 5-7 of a quarterly report for our ATP grant, covering October 2001 to December 2001, which reports our success in creating founder mice used to create transgenic mice (see especially page 7, bracketed paragraph).

Development of Improved Constructs for Optimized Expression

41. Between October 2000 and June 2001, we re-designed constructs to improve their performance. Point mutations were introduced into the thymidine kinase gene to prevent male sterility, which results from testicular expression of thymidine kinase. We also compared yeast and bacteria cytosine deaminase for use as suicide genes. The yeast version was selected based on its sensitivity to the 5-fluorouracil. **Exhibit 33** contains copies of pages 3-5 of a quarterly report covering November and December 2000, which identifies optimal suicide genes and promoters for liver-specific expression (see particularly the bracketed paragraph on page 3).

42. **Exhibit 34** is a copy of page 4 of the quarterly report for our ATP grant for January 2001 to March 2001 that describes making plasmids with cytosine deaminase as a suicide gene (see Figure 2).

43. **Exhibit 35** is a copy of page 4 of the quarterly report for our ATP grant for April 2001 to June 2001 that describes how we made the mutated thymidine kinase gene.

44. Between January 2001 and June 2001, we isolated and sequenced the porcine albumin promoter used for localized expression in the pig liver. We made plasmids to transfect the mutated thymidine kinase into fibroblasts. Nuclei from the transfected fibroblasts were injected into enucleated pig oocytes to make the transgenic pigs. **Exhibit 36** contains copies of laboratory notebook pages that describes experiments we performed to obtain the albumin promoter. Exhibit page 5 dated March 5, 2001 describes how we amplified the promoter. The associated picture shows the band of amplified promoter DNA. These experiments in **Exhibit 36** were carried out between February 22, 2001 and June 15 2001, for example on February 22, (**Exhibit 36**, page 1-2), February 27 (**Exhibit 36**, page 3), February 28 (**Exhibit 36**, page 2), March 2, 5 (**Exhibit 36**, page 5), March 5,6 (**Exhibit 36**, page 6), and between February and June 18, 2001 (**Exhibit 36**, pages 7-13).

45. **Exhibit 37** contains copies of pages 3-6 of a quarterly report for our ATP grant for January 2001 to March 2001. Page 3 documents our progress towards producing suicide gene constructs and provides the nucleotide sequences we determined of for the porcine albumin and alpha fetoprotein genes. Figure 1 is the Albumin promoter sequence. Figure 2 is the AFP promoter sequence. Pages 4-5 describe our synthesis of the constructs. Pages 5-6 describe our

efforts to develop stably transfected cell lines and our experiments infusing normal fetal pigs with human hepatocytes.

46. Between March 2001 and July 2001, we prepared constructs containing the AFP promoter and a suicide gene. **Exhibit 38** contains copies of pages 3-6 of a quarterly report for our ATP grant for the period April 2001 to June 2001 which summarizes development of the constructs. The bracketed paragraph on page 3 describes making a construct using the AFP promoter to drive cytosine deaminase expression (see Figure 2 on page 4). Page 4 describes a construct containing the SV40 enhancer and the porcine albumin promoter that controls expression of a mutated version of the thymidine kinase gene (see Figure 3). Page 5 describes a plasmid containing a mutated thymidine kinase gene under the control of a CMV promoter (see Figure 4). Pages 5-6 describe subcloning of plasmids into mammalian expression vectors for use in *in vitro* testing of the promoters.

47. **Exhibit 39** contains copies of laboratory notebook pages which document making the constructs. Page 35 is dated June 28, 2001 and shows a diagram of the construct using the AFP promoter to drive thymidine kinase expression. These experiments in **Exhibit 39** were carried out between March 2001 and June 2001, for example on June 17, (**Exhibit 39**, page 1), June 28 (**Exhibit 39**, pages, 2 3), June 26, 28 (**Exhibit 39**, page 5), March 15, 19 (**Exhibit 39**, page 6), March 20, 21 (**Exhibit 39**, page 7), (**Exhibit 39**, page 1), March 22 (**Exhibit 39**, page 8), March 23, 24 (**Exhibit 39**, page 9), April 2, 3 (**Exhibit 39**, page 11), April 4, 5, 6 (**Exhibit 39**, page 12) April 7 (**Exhibit 39**, page 13), April 16 (**Exhibit 39**, page 14), Between April 16 and March 23 (**Exhibit 39**, page 15), March 23 (**Exhibit 39**, page 6) May 1, (**Exhibit 39**, page

17), May 3, 4 (**Exhibit 39**, page 18), May 6 (**Exhibit 39**, page 19), May 7m 8 (**Exhibit 39**, page 20), May 9 (**Exhibit 39**, page 21), May 12, 16 (**Exhibit 39**, page 22), and May 18 (**Exhibit 39**, page 23).

48. Between March 2001 and September 2001, we transfected plasmids with the albumin and AFP promoters driving expression of thymidine kinase into the Huh-7 and TIB-73 cell lines. **Exhibit 40** contains copies of pages 4-5 of a quarterly report for our ATP grant covering July 2001 to September 2001. Figure 4 on page 4 is a Western blot that demonstrates thymidine kinase expression in TIB-73 cells under the control of the albumin promoter and in Huh-7 cells under the control of the AFP promoter. Figure 5 on page 5 is a graph that illustrates increased cell death in Huh-7 cells expressing thymidine kinase in response to gancyclovir treatment.

49. **Exhibit 41** contains pages 6 and 7 of a quarterly report for our ATP grant covering March 2001 to June 2001, which discusses results of our transfection experiments. Figure 6 at the bottom of page 6 shows thymidine kinase expressed in the Huh-7 cell line indicated by the presence of GFP. Pages 6-7 describe prodrug-killing assays using stably transfected cell lines. Page 7 reports our progress in infusion normal fetal pigs with human hepatocytes and prodrugs.

50. **Exhibit 42** contains laboratory notebook pages documenting the experiments summarized in **Exhibit 40** and **Exhibit 41**. The flow cytometry data starting on the page dated September 17, 2001 was used to make figure 5 in **Exhibit 41**. These experiments in **Exhibit 42** were carried out between June 2001 and September 2001, for example on June 28, (**Exhibit 42**,

page 1), July 31 (**Exhibit 42**, page 2), August 1 (**Exhibit 42**, page 3), August 3 (**Exhibit 42**, page 4), August 8 (**Exhibit 42**, page 5), August 10, 12 (**Exhibit 42**, page 6), between August 12 and September 7 (**Exhibit 42**, pages 7, 8, 9), September 14 (**Exhibit 42**, page 10), September 18 (**Exhibit 42**, page 11), September 12, 13 (**Exhibit 42**, page 12), between September 13 and September 17 (**Exhibit 42**, page 14 and 15) and September 25 (**Exhibit 42**, pages 16 and 17).

Creation of Transgenic Animals Containing a Suicide Gene

51. Between July 2001 and December 2001, we prepared and cloned fetal pig fibroblasts harboring plasmids in preparation for nuclear transfer. **Exhibit 43** is a copy of page 4 of the quarterly report for our ATP grant covering July 2001 to September 2001. Section 2, “Development of transgenic pigs,” describes how we made fibroblasts expressing thymidine kinase driven by the albumin promoter.

52. **Exhibit 44** is page 3 of a quarterly report for our ATP grant covering October 2001 to December 2001 and describes culturing and transfecting pig fibroblasts. Paragraphs 1 and 2 detail our experimental protocol used to make fibroblasts expressing thymidine kinase from the Albumin promoter.

53. From January 2002 to July 2002, we successfully bred mice homozygous for the suicide genes thymidine kinase and cytosine deaminase. **Exhibit 45** is a copy of page 4 of a quarterly report for our ATP grant covering January 2002 to March 2002. Paragraph 5 and the associated table shows that several mice in the F1 and F2 generation contained the transgene.

54. **Exhibit 46** is a copy of page 5 of a quarterly report for our ATP grant covering April 2002 to June 2002 which describes results of PCR experiments to identify mice homozygous for the transgene. Several samples in Figure 3 contained the 500 base pair PCR band, which indicates the presence of the transgene.

55. **Exhibit 47** contains copies of notebook pages which document results of histology experiments on mouse livers after addition of gancyclovir. The experiments in **Exhibit 47** were carried out between July 2001 and August 2001, for example on July 25, (**Exhibit 47**, page 1), August 12 (**Exhibit 47**, pages 2 and 3).

Creation Of Transgenic Pigs Containing The Mutated Thymidine Kinase Suicide Gene

56. From March 2002 to July 2002, we created a transgenic pig fetus. While the fetus did not survive, the liver expressed the mutated thymidine kinase gene. Thymidine kinase expression was driven by the albumin promoter, and we detected the expressed protein at 6 weeks gestation. **Exhibit 48** contains copies of pages 3 and 4 of a quarterly report for our ATP grant covering March 2002 to June 2002 and shows expression of the GFP marker in the fetal pig's liver (Figure 1 and the bracketed paragraph on page 3). Western blot analysis showed expression in the same fetus of thymidine kinase driven by the albumin promoter (Figure 2 on page 4).

57. From July 2002 to October 2002 we implanted more than eleven sows with nuclear transplanted embryos. Two male pigs were successfully delivered alive. Both expressed suicide genes in the liver. **Exhibit 49** contains copies of pages 3 and 4 of a quarterly report for

our ATP grant covering July 2002 to September 2002 and shows a PCR gel demonstrating the transgene is present in the piglets (Figure 1). Table 1 on page 4 describes anticipated births from additional embryo transfers.

58. **Exhibit 50** is a copy of a laboratory notebook page dated July 27, 2002, which describes nuclear transfer of embryos. **Exhibit 51** is a record of implanted embryos and ultrasound analysis of the fetuses.

59. On September 5, 2002 I asked Banner & Witcoff, Ltd. to a provisional patent application directed to the method described above (*i.e.*, a method of selectively destroying native cells in a tissue of a fetal non-human mammal host without substantially reducing the number of maternal cells of the same tissue and then implanting foreign replacement cells in the tissue of the fetal non-human mammal host). On September 12, 2002 I responded to questions from Banner & Witcoff about the draft application. On September 19, 2002 I approved the completed provisional application, which was filed the same day as provisional application No. 60/411,790. **Exhibit 52** contains copies of emails between Banner & Witcoff and me during this period.

60. All statements I made in this declaration of my own knowledge are true. I believe all statements made on information and belief to be true. I made these statements with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent.

Dated: April 17, 2009

_____/William E. Beschorner, M.D./____

William E. Beschorner, M.D.

CURRICULUM VITAE**William Edward Beschorner, M.D.****Current Appointments:**

Ximerex, Inc. President and Chief Scientific Officer
University Nebraska Medical Center Adjunct Professor of Surgery

Address:

2614 N. 161 Ave.
Omaha, Nebraska 68116-2461

Tel. (402) 426-0660, ext. 100
Fax: (402) 426-0663
Email: Beschorner@ximerex.com

Personal and Family

Date of Birth:	June 4, 1947		
Place of Birth:	Aurora, Illinois		
Marital Status:	Married, 1968		
Wife's Name:	Susan Mildred Beschorner		
Children:	Heather Elizabeth	Born:	1974
	William Frederick		1976
	Kurt Edward		1982

Education

1965-68	B.A., Augustana College Rock Island, Illinois
1968-71	M.S., Biochemistry St. Louis University St. Louis, Missouri
1973-76	M.D., University of Illinois Chicago, Illinois
1976-80	Intern / Resident Department of Pathology The Johns Hopkins Hospital Baltimore, Maryland

Certification

1977	Diplomate of National Board of Medical Examiners
1979	License, State of Maryland Board of Medical Examiners
1980	Certification, American Board of Pathology, Anatomic Pathology

Professional Experience

1971-73	Senior Research Biochemist Baxter/Travenol Labs. Morton Grove, Illinois
1980-81	Instructor, Departments of Pathology and Oncology The Johns Hopkins University School of Medicine Baltimore, Maryland
1981-86	Assistant Professor, Departments of Pathology and Oncology The Johns Hopkins University School of Medicine Baltimore, Maryland
1986-1997	Associate Professor Departments of Pathology and Oncology The Johns Hopkins University School of Medicine Baltimore, Maryland
1993-	Founder and President, Ximerex, Inc. Baldwin, Maryland
1997-2000	Professor of Surgery The University Nebraska Medical Center

Professional Activities:

The United States and Canadian Academy of Pathology, Inc.,
The American Association of Pathologists, Inc.
The Transplantation Society

Editorial Activities:

Ad hoc reviewer:
American Journal of Pathology
Clinical Immunology and Immunopathology
Bone Marrow Transplantation
Blood

Honors and Awards:

1982 Benjamin Castleman Award

Institutional Administrative Appointments:

1988-1993 Department of Pathology Space Committee
The Johns Hopkins University School of Medicine
1988-1993 Department of Pathology Research Resource Committee
The Johns Hopkins University School of Medicine
1981-2004 Board of Directors, Chairman of strategic planning
committee, Alpha Christian Registry, Inc.

Classroom Instruction:

1985-1995 Immunopathology, Year 2 Medical School Pathology
The Johns Hopkins University School of Medicine
1988-1994 Director, Immunopathology Fellowship Program
The Johns Hopkins University School of Medicine

NIH Scientific Review Board:

2002-Present SBIR Immunology Study Section
2005 Chair, Xenotransplantation RFA 2005

Clinical Attending Responsibilities:

1977-1995 Pathology Liaison with Bone Marrow Transplant Unit
The Johns Hopkins Hospital
1981-1993 Director, Immunophenotyping Laboratory
The Johns Hopkins Hospital

Patents

1. Method for Induction of Antigen-Specific Immune Tolerance, U.S. (No. 5, 597,563, 1/28/1997) and Canada (pending).
2. Surrogate Tolerogenesis for the Development of Tolerance to Xenografts, issued in U.S. (No. 6,060,049, 5/9/2000), issued Australia, Europe, pending in Canada, and Japan,.
3. Transplant Organs Accommodated Prior to Transplantation to be Resistant to Anti-Donor Immunity, PCT application, 1/25/01.
4. Growth of Foreign Cells after Conditional and Selective Destruction of Fetal Host Cells, PCT application, priority date 9/5/02.
5. Transgenic Pigs Producing Human Preproinsulin, PCT application, priority date 3/31/05

Sponsored Research Support:

Grant	Institution	Principal Investigator	Percent Effort
Bone Marrow Transplantation in Human Diseases, 1977-1993	NCI CA 15396 4 competitive renewals	G. W. Santos	25%
Chronic GVHD in the Rat Radiation Chimera, 1980-1991	NCI CA28701 Initial + 3 competitive renewals	W.E. Beschorner	40%
AIDS Cardiomyopathy, 1987-1992	NIAID HL 41514	A. Herskowitz	6%
AIDS Enteropathy, 1988-1991	NIDDK DK40618	J.G. Bartlett	6%
Treatment of Autoimmune Diseases by Sequential Foundation "Reeducation" of the thymus, 1992-1993	Mallinckrodt	W. E. Beschorner	10%
Induction of Xenograft Tolerance using Surrogate Tolerogenesis, 1993-1995	Ximerex, Inc.	W.E. Beschorner	25%
Surrogate Tolerogenesis for Xenotransplantation, 1995-1996	NIDDK, DK50737 SBIR, phase I	W.E. Beschorner	50%
Surrogate Tolerogenesis for Xenotransplantation, 1997-1999	NIDDK, DK50737 SBIR phase II	W.E. Beschorner	60%
Development of Hybrid Human/Pig Liver Xenografts, 2000	NIDDK, DK57986 SBIR, phase I	W.E. Beschorner	20%
Human/Pig Hybrid Livers for Transplantation, 2000-2003	ATP, NIST	W.E. Beschorner	100%
Human/ Pig Model of Hepatitis C Virus for New Vaccines, 2004-2006	NIH, AI058332 SBIR AT, phase I	W.E. Beschorner	10%
Heart Xenotransplantation with Chimeric Donor Pigs, 2005-2006	NIH HL079779 SBIR, phase I	W.E. Beschorner	25%
Islet Transplantation with Chimeric Donor Pigs, 2004-2007	NIH DK057986 SBIR, phase II	W.E. Beschorner	25%
Acceptance of Islet Xenografts in Primates using Chimeric Donor Pigs	JDRF 15-2005-800	W.E. Beschorner	25%

Immune Regulation in
Chimeric Donor Pigs

NIHDK057986-05 W.E. Beschorner 25%
SBIR, phase II competing continuation, 2007-10, pending

Articles Published in Peer Reviewed Journals

1. Stern, I. J., Izzo, R. S., Jo-Wang, Z. W., and Beschorner, W. E. Mechanisms in urea nitrogen binding by proposed oxidized starch gastrointestinal absorbents. *Experimentia* 31:1065-6, 1975.
2. Beschorner, W. E., Hutchins, G. M., Elfenbein, G. J., and Santos, G. W. The thymus in patients with allogeneic bone marrow transplants. *Am. J. Pathol.* 92:173-81, 1978.
3. Beschorner, W. E., Saral, R., Hutchins, G. M., Tutschka, P. J., and Santos, G. W. Lymphocytic bronchitis associated with graft-versus-host disease in bone marrow transplant recipients. *N. Engl. J. Med.* 299:1030-6, 1978.
4. Khouri, N. F., Saral, R., Armstrong, P. J., Santos, G. W., Beschorner, W. E., and Siegelman, S. S. Pulmonary interstitial changes following bone marrow transplantation. *Radiology* 133:587-92, 1979.
5. Tutschka, P. J., Santos, G. W., and Beschorner, W. E. The role of suppressor cells in transplantation tolerance. *Transplant. Proc.* 11:882-6, 1979.
6. Khouri, N., Saral, R., Armstrong, E. M., Tutschka, P. J., and Beschorner, W. E. Siegelman, S. Pulmonary interstitial changes following bone marrow transplantation. *Radiol.* 133:587-90, 1979.
7. Tutschka, P. J., Beschorner, W. E., Allison, A. C., Burns, W. H., and Santos, G. W. Use of Cyclosporin A in allogeneic bone marrow transplantation (BMT) in the rat. *Nature* 280:148-51, 1979.
8. Beschorner, W. E., Hess, A. D., Nerenberg, S. T., and Epstein, R. B. Isolation and characterization of canine venereal tumor associated inhibitory and blocking factors. *Cancer Res.* 39:3920-7, 1979.
9. Tutschka, P. J., Beschorner, W. E., and Hess, A. D. Use of Cyclosporin A (CsA) to prevent graft-versus-host disease (GVHD) in a rat model of allogeneic marrow transplantation. *Hamatol. Bluttransfus.* 25:241-53, 1980.
10. Beschorner, W. E., Pino, J., Boitnott, J. K., Tutschka, P. J., and Santos, G. W. Pathology of the liver with bone marrow transplantation: Effects of busulfan, carmustine, acute graft-versus-host disease and cytomegalovirus infection. *Am. J. Pathol.* 99:369-85, 1980.
11. Beschorner, W. E., Hutchins, G. M., Burns, W. H., Saral, R., Tutschka, P. J., and Santos, G. W. Cytomegalovirus pneumonia in bone marrow transplant recipients: Miliary and diffuse patterns. *Am. Rev. Resp. Dis.* 122:107-14, 1980.
12. Tutschka, P. J., Elfenbein, G. J., Sensenbrenner, L. L., Saral, R., Kaizer, H., Order, S. E., Beschorner, W. E., Farmer, E., and Santos, G. W. Preparative regimens for marrow transplantation in acute leukemia and aplastic anemia - Baltimore experience. *Am. J. Hematol. Oncol.* 2:363-70, 1980.
13. Tutschka, P. J., Korbiling, M., Hess, A. D., and Beschorner, W. E. Prevention of graft-versus-host disease (GVHD) by chemoseparation of marrow cells. *Transplant. Proc.* 13:1202-6, 1981.
14. Beschorner, W. E., Yardley, J. H., Tutschka, P. J., and Santos, G. W. Deficiency of intestinal immunity with graft-vs.-host disease in humans. *J. Infect. Dis.* 144:38-46, 1981.
15. Tutschka, P. J., Hess, A. D., Beschorner, W. E., and Santos, G. W. Suppressor cells in transplantation tolerance. I. Suppressor cells in the mechanism of tolerance in radiation chimeras. *Transplantation* 2:203-9, 1981.

16. Tutschka, P. J., Ki, P. F., Beschorner, W. E., Hess, A. D., and Santos, G. W. Suppressor cells in transplantation tolerance. II. Maturation of suppressor cells in the bone marrow chimera. *Transplantation* 32:321-5, 1981.
17. Beschorner, W. E., Tutschka, P.J., and Santos, G. W. Sequential morphology of acute graft-versus-host disease in the rat radiation chimera. *Clin. Immunol. Immunopathol.* 22:203-24, 1982.
18. Beschorner, W. E., Tutschka, P. J., and Santos, G. W. Chronic graft-versus-host disease in the rat radiation chimera. I. Clinical features, hematology, histology, and immunopathology in long term chimeras. *Transplantation* 33:393-97, 1982.
19. Tutschka, P. J., Hess, A. D., Beschorner, W. E., and Santos, G. W. Suppressor cells in transplantation tolerance. III. The role of antigen in the maintenance of transplantation tolerance. *Transplantation* 33:510-4, 1982.
20. Tutschka, P. J., Teasdale, R., Beschorner, W. E., and Santos, G. W. Chronic graft-versus-host disease in rat radiation chimera. II. Immunologic evaluation in long term chimera. *Transplantation* 34:289-94, 1982.
21. Townsend, T. R., Bolyard, E. A., Yolken, R. N., Beschorner, W. E., Bishop, C. A., Santos, G. W., and Saral, R. An outbreak of coxsackie A1 gastroenteritis: A complication of bone marrow transplantation. *Lancet* I:820-3, 1982.
22. Belanger, R., Tutschka, P. J., Beschorner, W. E., and Santos, G. W. A Santos, G. W. Acute graft-versus-host disease in recipients of syngeneic bone marrow. *Exp. Hematol.* 10:23-32, 1982.
23. Beschorner, W. E., Tutschka, P. J., and Santos, G. W. Chronic graft-versus-host disease in the rat radiation chimera. III. Immunology and immunopathology in rapidly induced models. *Transplantation* 35:224-30, 1983.
24. Tutschka, P. J., Beschorner, W. E., Hess, A. D., and Santos, G. W. Cyclosporin A to prevent graft-versus-host disease. A pilot study in 22 patients receiving allogeneic marrow transplants. *Blood* 61:318-25, 1983.
25. Tutschka, P. J., Belanger, R., Hess, A. D., Beschorner, W. E., and Santos, G. W. Suppressor cells in transplantation tolerance to self and non-self. *Transplant. Proc.* 15:853-8, 1983.
26. Jabs, D. A., Hirst, L. W., Green, W. R., Tutschka, P. J., Santos, G. W., and Beschorner, W. E. The eye in bone marrow transplantation. II. Histopathology. *Arch. Ophthalmol.* 101:585-90, 1983.
27. Tutschka, P. J., Hess, A. D., Beschorner, W. E., Farmer, E. R., and Santos, G. W. Cyclosporin A in clinical marrow transplantation - Baltimore experience. *Transplant. Proc.* 15:2613-6, 1983.
28. Santos, G. W., Tutschka, P. J., Brookmeyer, R., Saral, R., Beschorner, W. E., Bias, W. B., Braine, H. G., Burns, W. H., Elfenbein, G. J., Kaizer, H., Mellits, D., Sensenbrenner, L. L., Stuart, R. K., and Yeager, A. M. Marrow transplantation for acute non-lymphocytic leukemia (ANL) following treatment with busulfan (BU) and Cyclophosphamide (Cy). *N. Engl. J. Med.* 309:1347-53, 1983.
29. Haupt, H. M., Merz, W. G., Beschorner, W. E., Vaughan, W. P., and Saral, R. Colonization and infection with *Trichosporan* Species in the immuno-suppressed host. *J. Infect. Dis.* 147:199-203, 1983.
30. Bross, D. S., Tutschka, P. J., Farmer, E. R., Beschorner, W. E., Braine, H. G., Mellits, E. D., Bias, W. B., and Santos, G. W. Predictive factors for acute graft-versus-host disease in patients transplanted with HLA identical bone marrow. *Blood* 63:1265-70, 1984.
31. Bender, W. L., Whelton, A., Beschorner, W. E., Darwish, M. O., Hall-Craggs, M., and Solez, K. Interstitial nephritis, proteinuria, and renal failure caused by nonsteroidal anti-inflammatory drugs. Immunologic characterization of the inflammatory infiltrate. *Am. J. Med.* 76:1006-12, 1984.

32. Beschorner, W. E. Destruction of the intestinal mucosa after bone marrow transplantation and graft-versus-host disease. *Surv. Synth. Pathol. Res.* 3:264-74, 1984.
33. Burdick, J. F., Beschorner, W. E., Smith, W. J., McGraw, D., Bender, W. L., Williams, G. M., and Solez, K. Characteristics of early routine renal allograft biopsies. *Transplantation* 38:679-84, 1984.
34. Burdick, J. F., McGraw, D., Bender, W., Beschorner, W. E., Williams, G. M., and Solez, K. Renal allograft infiltrate in the absence of rejection. *Transplant. Proc.* 16:1580-2, 1984.
35. Parfrey, N. A., Mann, R. B., Selonick, S. E., and Beschorner, W. E. Malignant large cell lymphoma of B-cell type with multilobated nuclei. Report of a case and review of the literature. *Cancer* 55:1913-7, 1985.
36. O'Kunewick, J. P., Beschorner, W. E., Buffo, M. J., and Kociban, D. L. Histopathology of a possible graft-versus-host reaction induced by nude mouse spleen cells. *Transplantation* 39:447-9, 1985.
37. Patchell, R. A., White, C. L. III, Clark, A. W., Beschorner, W. E., and Santos, G. W. Nonbacterial thrombotic endocarditis in bone marrow transplant recipients. *Cancer* 55:631-5, 1985.
38. Beschorner, W. E., Burdick, J. F., Williams, G. M., and Solez, K. The presence of Leu 7 reactive lymphocytes in renal allografts undergoing acute rejection. *Transplant. Proc.* 17:618-22, 1985.
39. Kaizer, H., Stuart, R. K., Brookmeyer, R., Beschorner, W. E., Braine, H. G., Burns, W. H., Fuller, D. J., Korbling, M., Mangan, K. F., Saral, R., Sensenbrenner, L., Shadduck, R. K., Shende, A. C., Tutschka, P. J., Yeager, A. M., Zinkham, W. H., Colvin, O. M., and Santos, G. W. Autologous bone marrow transplantation (BMT) in acute leukemia: a phase I study of in vitro treatment of marrow with 4-hydroperoxycyclophosphamide (4HC) to purge tumor cells. *Blood*. 65:1504, 1985.
40. Patchell, R. A., White, C. L. III, Clark, A. W., and Beschorner, W. E. Neurologic complications complications of bone marrow transplantation. *Neurology*. 35:300-6, 1985.
41. Burdick, J. F., Beschorner, W. E., Smith, W. J., McGraw, D., Bender, W. L., Williams, G. M., and Solez, K. Lymphocytes in early routine renal allograft biopsies. *Transplant. Proc.* 17:562-3, 1985.
42. Beschorner, W. E., Civin, C. I., and Strauss, L. C. Localization of hematopoietic progenitor cells in tissue with the anti-My-10 monoclonal antibody. *Am. J. Pathol.* 119:1-4, 1985.
43. Hess, A. D., Horwitz, L., Beschorner, W. E., and Santos, G. W. Development of graft-vs-host disease like syndrome in Cyclosporine-treated rats after syngeneic bone marrow transplantation. I. Development of cytotoxic T lymphocytes with apparent polyclonal anti-Ia specificity, including autoreactivity. *J. Exp. Med.* 161:718-730, 1985.
44. Solez, K., McGraw, D. J., Beschorner, W. E., Kone, B. C., Racusen, L. C., Whelton, A., and Burdick, J. F. Reflections on use of the renal biopsy as the "gold standard" in distinguishing transplant rejection from Cyclosporine nephrotoxicity. *Transplant. Proc.* 17:123-33, 1985.
45. Levy, D. A., Wefald, A. F., and Beschorner, W. E. Intestinal mast cell proliferation in graft-versus-host reaction in rats. *Int. Arch. Allergy Appl. Immunol.* 77:186-8, 1985.
46. Ueda, K., Baumgartner, W. A., Beschorner, W. E., Borkon, M. A., Soule, L. M., Reitz, B. R., Herskowitz, A. Histologic patterns of early cardiac rejection in allografts with Cyclosporine treatment. *J. Heart Transplant.* 4:296-301, 1985.
47. Hymes, S. R., Simonton, S. C., Farmer, E. R., Beschorner, W. E., Tutschka, P. J., and Santos, G. W. Cutaneous busulfan effect in patients receiving bone-marrow transplantation. *J. Cutan. Pathol.* 12:125-9, 1985.

48. Ambinder, R., Burns, W., Forman, M., Charache, P., Beschorner, W., Santos, G., and Saral, R. Hemorrhagic cystitis associated with adenovirus infection in a bone marrow transplant recipient. *Arch. Intern. Med.* 146:1400-1, 1986.
49. McDonnell, J. M., Beschorner, W. E., Staal, S. P., Spivak, J. L., and Mann, R. B. Richter's Syndrome with Two Different B Cell Clones. *Cancer*, 58:2031-7, 1986.
50. Jones, B., Fishman, E. K., Kramer, S. S., Siegelman, S. S., Saral, R., Beschorner, W. E., Yeager, A. M., Lake, A. M., Yolken, R. H., Tutschka, P., Santos, G. W. Computed tomography of gastrointestinal inflammation after bone marrow transplantation. *Am. J. Roentgenology* 146:691-695, 1986.
51. Simons, J. W., Noga, S. J., Colombani, P. M., Beschorner, W. E., Coffey, D. S., and Hess, A. D. Cyclosporine A an in vitro calmodulin antagonist induces nuclear lobulations in human T-cell lymphocytes and monocytes. *J. Cell Biol.* 102:145-50, 1986.
52. McDonnell, J. M., Beschorner, W. E., Kuhajda, F. P., and deMent, S. H. Common leukocyte antigen staining of a primitive sarcoma. *Cancer*, 59:1438-41, 1987.
53. Beschorner, W. E., Namnoum, J.D., Hess, A. D., Shinn, C.A., and Santos, G. W. Cyclosporin A and the Thymus. *Immunopathology. Am. J. Pathol.*, 126:487-496, 1987.
54. Robey, S. S., Caferty, L. L., Beschorner, W. E., and Gupta, P.G. Value of Lymphocyte Marker Studies in Diagnostic Cytopathology, *Acta Cytologica*, 31:453-9, 1987.
55. Beschorner, W. E., Namnoum, J. D., and Hess, A. D. Immunopathology of rat thymus after Cyclosporin A. *Transplant. Proc.*, 19:1230-5, 1987.
56. Beschorner, W. E., Farmer, E. R., Saral, R., Stirling, W. L., and Santos, G. W. Epithelial class II antigen expression in cutaneous GVHD. *Transplantation*, 44:237-43, 1987.
57. Hess, A. D., Vogelsang, G. B., Heyd, J., and Beschorner, W. E. Cyclosporine induced syngeneic graft-versus-host disease: Assessment of T-cell differentiation. *Transplant. Proc.*, 19:2683-6, 1987.
58. Jones, R. J., Lee, K. S. K., Beschorner, W. E., Vogel, V. G., Grochow, L. B., Braine, H. G., Vogelsang, G. B., Sensenbrenner, L. L., Santos, G. W., and Saral, R. Veno-occlusive disease of the liver following bone marrow transplantation. *Transplantation* 44:778-83, 1987.
59. Santos, G. W., Tutschka, P. J., Brookmeyer, R., Saral, R., Beschorner, W. E., Bias, W. B., Braine, H. G., Burns, W. H., Farmer, E.R., Hess, A. D., Kaizer, H., Mellits, D., Sensenbrenner, L. L., Stuart, R., and Yeager, A. M. Cyclosporine plus methylprednisolone as prophylaxis for graft-versus-host disease: A randomized double blind study in patients undergoing allogeneic marrow transplantation. *Clin. Transplantation* 1:21-8, 1987.
60. Fishel, R.S., Barbul, A, Beschorner, W.E., Wasserkrug, H.L, and Efron, G. Lymphocyte participation in wound healing: morphologic assessment using monoclonal antibodies. *Ann. Surgery*, 206:25-9, 1987.
61. Beschorner, W.E., Di Gennaro, K.A., Hess, A.D., and Santos, G.W. Cyclosporine (CsA) and the thymus. Influence of irradiation and age on thymic immunopathology and recovery. *Cell Immunol.* 110:350-64, 1987.
62. Beschorner, W. E., Olson, J. L., Hess, A. D., DiGennaro, K. A., and Santos, G. W. Cyclosporine-induced cell mediated injury of the thymic medullary epithelium. *Transplantation* 45:797-803, 1988.
63. Sliman, G. A., Beschorner, W. E., Baughman, K., Hutchins, G. M., Borkon, M. A., and Baumgartner, W. A. Graft-versus-host disease in a heart allograft recipient. A possible autoimmune phenomenon. *Transplantation*, 45:253-6, 1988.

64. Beschorner, W. E., Hess, A. D., Shinn, C. A., and Santos, G. W. Transfer of Cyclosporin A associated syngeneic graft-vs-host disease (GVHD) by thymocytes. Resemblance to chronic GVHD. *Transplantation*, 45:209-15, 1988.
65. Beschorner, W.E., Shinn, C.A., Fischer, A.C., Santos, G.W., and Hess, A.D. Cyclosporine (CsA) induced pseudo graft-versus-host disease (CIPGVHD) in the early post CsA period. *Transplantation* 46:112S-117S, 1988.
66. Herman, J.G., Beschorner, W.E., Baughman, K.L., Boitnott, J.K., Vogelsang, G.B., and Baumgartner, W.A. Pseudo Graft-vs-Host Disease in Heart and Heart-Lung Recipients, *Transplantation* 46:93-8, 1988.
67. Beschorner, W.E., Suresch, D. L., Shinozawa, T., Santos, G.W., and Hess, A.D. Thymic Immunopathology After Cyclosporine: Effect of Irradiation and Age on Medullary Involution and Recovery. *Transplant Proc.* 20:1072-8, 1988.
68. Hess, A.D., Vogelsang, G.B., Silanskis, M., Friedman, K.A., Beschorner, W., and Santos, G.W. Syngeneic graft-versus-host disease after allogeneic bone marrow transplantation and Cyclosporine treatment. *Transplant Proc.* 20:487-92, 1988.
69. Fischer, A.C., Beschorner, W.E., and Hess, A.D. Syngeneic graft-versus-host disease: Failure of autoregulation in self/non-self discrimination. *Transplant Proc.* 20:493-500, 1988.
70. Hruban, R.H., Beschorner, W.E., Gupta, P.K., Baumgartner, W.A., Achuff, S.C., Traill, T.A., Reitz, B.A., and Hutchins, G.M. Diagnosis of rejection of lung allograft. *N. Engl. J. Med.* 318:1129, 1988.
71. Hruban, R.H., Beschorner, W.E., Gupta, P.K., Baumgartner, W.A., Achuff, S.C., Traill, T.A., Reitz, B.A., and Hutchins, G.M. Diagnosis of lung allograft rejection by bronchial intraepithelial Leu-7 positive T lymphocytes, *J. Thor. Cardiovasc. Surgery*, 96:939-46, 1988.
72. Hruban, R.H., Beschorner, W.E., Baumgartner, W.A., Achuff, S.C., Traill, T.A., DiGennaro, K.A., Reitz, B.A., and Hutchins, G.M. Depletion of bronchial immunity associated with lung allograft rejection. *Am. J. Pathol.* 132:6-11, 1988.
73. Jones, B., Kramer, S.S., Saral, R., Beschorner, W.E., Yolken, R.H., Townsend, T.R., Yeager, A.M., Lake, A., Tutschka, P.J., Santos, G.W. Gastrointestinal inflammation after bone marrow transplantation: Graft-versus-host disease or opportunistic infection? *Am. J. Roentgenology*. 150:277-281, 1988.
74. Wingard, J.R., Mellits, E.D., Sostrin, M.B., Chen, D.Y-H, Burns, W.H., Santos, G.W., Vriesendorp, H.M., Beschorner, W.E., and Saral, R. Interstitial pneumonitis after allogeneic bone marrow transplantation: Nine year experience at a single institution. *Medicine*, 67:175-186, 1988.
75. Holland, K., Wingard, J.R., Beschorner, W.E., Santos, G.W., and Saral, R. Bronchiolitis obliterans in bone marrow transplantation and its relationship to chronic graft-v-host disease and low serum IgG. *Blood*. 72:621-627, 1989.
76. Staal, S.P., Ambinder, R., Beschorner, W.E., Hayward, G.S., and Mann, R. A survey of Epstein-Barr virus DNA in lymphoid tissue: it is present with high frequency in Hodgkins disease. *Amer. J. Clin. Pathol.* 91:1-5, 1989.
77. Fischer, A.C., Hess, A.D., and Beschorner, W.E. Age-related factors in the induction of syngeneic GVHD. *Transplant Proc.* 21:3033-3035, 1989.
78. Beschorner, W.E., Shinn, C.A., Hess, A.D., Suresch, D.L., and Santos, G.W. Immune related injury to endothelium associated with acute graft-vs-Host Disease in the rat. *Transplant. Proc.* 21:3025-3027, 1989.

79. Fischer, A.C., Beschorner, W.E., and Hess, A.D. Requirements for the induction and adoptive transfer of Cyclosporine induced syngeneic graft-versus-host disease. *J. Exp. Med.* 169:1031-1041, 1989.
80. Hess, A.D., Fischer, A.C., and Beschorner, W.E. Regulation of syngeneic graft-vs-host disease by autosuppressor mechanisms. *Transplant. Proc.* 21:3013-3015, 1989.
81. Wagner, J.E., Vogelsang, G.B., and Beschorner, W.E. The pathogenesis and pathology of graft-versus-host disease. *J. Ped. Hematol. Oncol.* 11:196-212, 1989.
82. Guyer, D.R., Jabs, D.A., Brant, A.M., Beschorner, W.E., and Green, W.R. Regression of cytomegalovirus retinitis with Zidovudine: A clinical/pathologic correlation. *Arch. Ophthalmol.* 107:868-874, 1989.
83. Geller, R.B., Esa, A.H., Beschorner, W.E., Frondoza, C.G., Santos, G.W., and Hess, A.D. Successful in vitro graft-vs-tumor effect against an Ia-bearing tumor using cyclosporine-induced syngeneic graft-vs-host disease in the rat. *Blood* 74:1165-71, 1989.
84. Wingard, J.R., Mellits, E.D., Jones, R.J., Beschorner, W.E., Sostrin, M.B., Burns, W.H., Santos, G.W., Saral, R. Association of hepatic veno-occlusive disease with interstitial pneumonitis in bone marrow transplant recipients. *Bone Marrow Transplantation* 4:685-689, 1989.
85. Horn, T.D., Redd, J.V., Karp, J.E., Beschorner, W.E., Burke, P.J., and Hood, A.F. The cutaneous eruption of lymphocyte recovery. *Arch. Dermatol.* 125:1512-1517, 1989.
86. Wingard, J.R., Piantadosi, S., Vogelsang, G.B., Farmer, E.R., Jabs, D.A., Levin, L.S., Beschorner, W.E., Cahill, R.A., Miller, D.F., Harrison, D., Saral, R., and Santos, G.W. Predictors of death from chronic graft versus host disease after bone marrow transplantation. *Blood* 74:1428-1435, 1989.
87. Dumler, J.S., Beschorner, W.E., Farmer, E.R., and Santos, G.W. Endothelial cell injury in cutaneous graft-vs-host disease. *Am. J. Pathology*, 135:1097-1103, 1989.
88. Fischer, A.C., Laulis, M.K., Horwitz, L., Beschorner, W.E., and Hess, AD. Host resistance to Cyclosporine induced syngeneic graft-versus-host disease: Requirement for two distinct lymphocyte subsets. *J. Immunol.* 143: 827-832, 1989.
89. Geller, R.B., Saral, R., Piantadosi, S., Zahurak, M., Vogelsang, G.B., Wingard, J.R., Ambinder, R.F., Beschorner, W.E., Braine, H.G., Burns, W.H., Hess, A.D., Jones, R.J., May, W.S., Rowley, S.D., Wagner, J.E., Yeager, A.M., and Santos, G.W. Allogeneic bone marrow transplantation following high-dose busulfan and cyclophosphamide in patients with AML. *Blood* 73:2209-2218, 1989.
90. Holland, H.K., Saral, R., Rossi, J. J., Donnenberg, A. D., Burns, W. H. Beschorner, W. E., Farzadegan, H., Jones, R. J., Quinnan, G. V., Vogelsang, G. B., et al. Allogeneic bone marrow transplantation, zidovudine, and human immunodeficiency virus type 1 (HIV-1) infection. Studies in a patient with non-Hodgkin lymphoma. *Ann. Intern. Med.* 111:973-81, 1989.
91. Herskowitz, A., Ahmed-Ansari, A., Neumann, D. A., Beschorner, W. E., Rose, N. R., Soule, L. M., Burek, C. L., Sell, K. W., and Baughman, K. L. Induction of major histocompatibility complex antigens within the myocardium of patients with active myocarditis: A nonhistologic marker of myocarditis. *J. Am. Coll. Cardiol.* 15:624-32, 1990.
92. MacCumber, M. W., Hoffman, P. N., Wand, G. S., Epstein, J. I., Beschorner, W. E., and Green, W. R. Ophthalmic involvement in aggressive histiocytosis X. *Ophthalmology* 97:22-27, 1990.
93. Moses, R.D., Beschorner, W.E., Singer, D., Maguire, J.E., Orr, K.S., Bacher, J.D., and Gress, R.E. Restriction fragment assessment of graft-versus-host disease, and cardiac allograft survival in rhesus monkeys conditioned with mixed MHC-mismatched donor plus autologous marrow transplantation. *Bone Marrow Transplantation* 4:475-81, 1990.

94. Hruban, R.H., Beschorner, W.E., Baumgartner, W.A., Marsh, B.R., Traill, T.A., Achuff, S.C., Ren, H., Reitz, B.A., and Hutchins, G.M. Evidence that the expression of class II MHC antigens is not diagnostic of lung allograft rejection. *Transplantation* 48:529-30, 1990.
95. Wingard, J. R., Piantadosi, S., Santos, G. W., Saral, R., Vriesendorp, H. M., Yeager, A. M., Burns, W. H., Ambinder, R. F., Braine, H. G., Elfenbein, G., Jones, R. J., Kaizer, H., May, W. S., Rowley, S. D., Sensenbrenner, L. L., Stuart, R. K., Tutschka, P. J., Vogelsang, G. B., Wagner, J. E., Beschorner, W. E., Brookmeyer, R., and Farmer, E. R. Allogeneic and bone marrow transplantation for patients with high-risk acute lymphoblastic leukemia. *J. Clin. Oncol.* 8:820-830, 1990.
96. Shinozawa, T., Beschorner, W.E., and Hess, A.D. Prolonged administration of Cyclosporine (CsA) and the thymus. Irreversible immunopathologic changes associated with autologous pseudo-graft-vs-host disease (GVHD). *Transplantation*, 50:106-111, 1990.
97. Hess, A. D., Fischer, A. C., and Beschorner, W. E. Effector mechanisms in Cyclosporine A-induced syngeneic graft-versus-host disease. Role of CD4+ and CD8+ T lymphocyte subsets. *J. Immunol.* 145:526-33, 1990.
98. Hruban, R. H., Wu, T-C, Beschorner, W. E., Cameron, D. E., Ambinder, R. F., Baumgartner, W. A., Reitz, B. A., Hutchins, G. M. Cytomegalovirus nucleic acids in allografted hearts. *Human Pathol.* 21:981-3, 1990.
99. Scherer, L.R.3d, Beschorner, W.E., Colombani, P.M. The ontogeny of the gut-associated lymphoid tissue in short bowel syndrome. *J. Surg. Res.* 48:358-62, 1990.
100. Funkhouser, A., Vogelsang, G., Zehnbaauer, B., Tunnessen, W. W., Beschorner, W. E., Sanders, M., and Graeber, J. Graft-versus-host disease after blood transfusions in a premature infant. *Pediatrics* 87:247-50, 1991.
101. Beschorner, W. E., Baughman, K., Turnicky, R. P., Hutchins, G. M., Rowe, S. A., Kavanaugh-McHugh, A.L., Suresch, D. L., Herskowitz, A. HIV associated myocarditis: pathology and immunopathology. *Am. J. Pathol.*, 137:1365-71, 1990.
102. Hruban, R.H., Beschorner, W. E., Baumgartner, W. A., Augustine, S. M., Ren H, Reitz, B. A., Hutchins, G. M. Accelerated arteriosclerosis in heart transplant recipients is associated with a T-lymphocyte mediated endothelialitis. *Am. J. Pathol.* 137: 871-82, 1990.
103. Beschorner, W.E. and Armas, O.A. Loss of medullary dendritic cells in the thymus after cyclosporine and irradiation, *Cell. Immunol.* 132:505-514, 1991.
104. Turnicky, R.P., Goodin, J., Smialek, J.E., Herskowitz, A., Beschorner, W.E. Incidental myocarditis with intravenous drug abuse: The pathology, immunopathology and possible relationship to HIV associated myocarditis. *Human Pathol.* 23:138-43, 1992.
105. Hruban, R.H., Beschorner, W.E., Baumgartner, W.A., Augustine, S.M., Reitz, B.A., Hutchins, G.M. Accelerated arteriosclerosis in heart transplant recipients: An immunopathology study of 22 transplanted hearts. *Transplant. Proc.* 23:1230-2, 1991.
106. Xu, R., Kittur, D.S., Burdick, J.F., Beschorner, W., and Adler, W. Relevance of actin gene expression in murine cardiac allografts. Submitted.
107. Xu, R., Burdick, J.F., Scott, A., Beschorner, W.E., Adler, W., and Kittur, D.S. Graft-specific MHC class II gene expression in response to allogeneic stimulus in heterotopic murine cardiac allografts. *Immunology*, 75:361-5, 1992.
108. Beschorner, W.E., Ren, H., Phillips, J., Pulido, H.B., Hruban, R.H., and Hess, A.D. Reconstitution of thymus dependent host resistance after cyclosporine prevents syngeneic GVHD., *Transplantation* 52:668-74, 1991.

109. Greenson, J.K., Beschorner, W. E., Boitnott, J. K., Yardley, J.H. Prominent mononuclear cell infiltrate is characteristic of herpes esophagitis, *Human Pathology* 22:541-9, 1991.
110. Beschorner, W.E., Divic, J., Pulido, H., Yao, X., Kenworthy, P., and Bruce, G. Thymic recovery after cyclosporine (CsA) is enhanced by human recombinant growth hormone (rhGH) and insulin-like growth factor I (rhIGF-1). *Transplantation* 52:879-84, 1991.
111. Griffin, C.A., Zehnbauser, B.A., Beschorner, W.E., Ambinder, R., and Mann, R. t(11;18)(q21;q21) is a recurrent chromosome abnormality in small lymphocytic lymphoma. *Genes Chromosom Cancer* 4:153-7, 1992.
112. Vogelsang, G.B., Farmer, E.R., Hess A.D., Altamonte V, Beschorner, W.E., Jabs, D.A., Corio, R.L., Levin L.S., Colvin O.M., Wingard J.R., Santos G.W. Thalidomide for the treatment of chronic graft-versus-host disease. *N. Engl. J. Med.* 326:1055-8, 1992.
113. Wu, T.C., Pizzorno, M.C., Hayward, G.S., Willoughby, S., Neumann, D.A., Rose, N.R., Ansari, A.A., Beschorner, W.E., Baughman, K.L., and Herskowitz, A. In situ detection of human cytomegalovirus immediated-early gene transcripts within cardiac myocytes of patients with HIV-associated cardiomyopathy. *AIDS.* 6:777-85, 1992.
114. Fox III, W.M., Hameed, A., Hutchins, G.M., Reitz, B.A., Baumgartner, W.A., Beschorner, W.E., and Hruban, R.H. Perforin expression localizing cytotoxic lymphocytes in the intimas of coronary arteries with transplant-related accelerated arteriosclerosis. *Human Pathology.* 24:477-82, 1993.
115. Wojno K.J., Beschorner, W.E., and Santos, G.W. Pulmonary hemorrhage as a cause of death in allogeneic bone marrow recipients with severe acute graft-vs-host disease. *Transplantation* 57:88-92, 1994.
116. Herskowitz A., Wu T.-C., Willoughby, S.B., Vlahov, D., Ansari, A.A., Beschorner, W.E., and Baughman, K.L. Myocarditis and cardiotropic viral infection associated with severe ventricular dysfunction in late-stage infection with human immunodeficiency virus. *J. Amer. Coll. Cardiology.* 24:1025-32, 1994.
117. Fischer, A.C., Ruvolo, P.P., Burt, R., Horwitz, L.R., Bright, E.C., Hess, J.M., Beschorner, W.E., and Hess AD. Characterization of the autoreactive T cell repertoire in cyclosporin-induced syngeneic graft-versus-host disease. A highly conserved repertoire mediates autoaggression. *J. Immunol.* 154: 3713-25, 1995.
118. Beschorner, W.E., Yao, X., Divic, J. Recruitment of allogeneic dendritic cells to the thymus during post-cyclosporine thymic regeneration, *Transplantation* 60: 1326-1330, 1995.
119. Beschorner, W.E., Qian, Z., Mattei, P., Hess, A.D., Colombani, P.M., Sciscione, A.C., Khouzami, A., Blakemore, K.J., and Burdick, J.F. Induction of human chimerism and functional suppressor cells in fetal pigs: feasibility of surrogate tolerogenesis for xenotransplantation. *Transplant. Proc.* 28:648-9, 1996.
120. Lomis, T.J., Hess, A.D., Blakemore, K.J., Lynd, C., Hall, S., Paletta, J. and Beschorner, W.E. Novel Process for inducing specific immune tolerance to pig xenografts without severe immune suppression. *Surgical Forum* 47:411-3, 1996.
121. Fox, R.J., Vogelsang, G.B., and Beschorner, W.E. Denuded bowel after recovery from graft-versus-host disease. *Transplantation* 62:1681-4, 1996.
122. Sudan, D.L., Radio, S.J., Matamoros, A., Johnson, N.D., Shearon, C.C., Glogowski K.R., Hill, A.C., Goertzen, T.C., Fox, I.J., Langnas, A.N., and Beschorner, W.E. Effect of surrogate tolerogenesis on the vascular rejection of pig heart xenografts. *Transplantation*, 69:232-5, 2000.

123. Beschorner, W., Sudan, D., Yang, T., Radio, S., Stammers, A., Johnson, N., Shearon, C., Kuszynski, C., Dixon, R.S., Matamoros Jr., A., Langnas A. Surrogate Tolerogenesis: Possible Pretransplant Induction of Accommodation of Pig Xenografts, *Transplant. Proc.* 32:994-5, 2000.
124. Yang, T., Stammers, A., Jiang, J., Shearon, C., Thompson, S., and Beschorner, W. E. Chimeric pig hearts resist hyperacute rejection in ex vivo perfusion model. *J.Extra.Corpor.Technol.* 33:181-184. 2001.
125. Xu, R., Burdick, J. F., Beschorner, W., Wilasrusmee, C., and Kittur, D. S. Expression of fetal isoforms of actin after transplantation injury. *Transpl.Int.* 15:296-301, 2002.
126. Beschorner, W.E., Sudan, D.L., Radio, S.J., Yang, T., Franco, K.L., Hill, A.C., Shearon, C.C., Thompson, S.C., Dixon, R.S., Johnson, N.D., Kuszynski, C.A., Rubocki, R.J., Lechtenberg, K.F., Matamoros, A., Jr., Goertzen, T.C., Fox, I.J., and Langnas, A.N. Heart Xenograft Survival with Chimeric Pig Donors and Modest Immune Suppression, *Annals of Surgery* 237:265-72, 2003.
127. Beschorner, W.E., Shearon, C.C., Yang, T., Langnas, A.N., Thompson, S.C., Zhao, Y., Franco, K.L. and Sudan, D.L. Pretransplant Analysis of Accommodation in Donor Pigs, *Xenotransplantation* 10:66-71, 2003.
128. Beschorner, W.E. Xenotransplantation, *eMedicine Review*, 2006
129. Stevens, R.B., Rigley, T.H., Skorupa, J.Y., Miller, J.D., Lui G., Botha, J.F., Veys, T.J., Nielson, K., Henning, M., Wisecarver, J.L., Kuszynski C.A., Wilkie, L.M., Harbour-Marik, V.K., Van Winkle, S.A., Bills, N.D., Lechtenberg, K.F., Schieber, T.L., Langnas, A.N., Beschorner, W.E. Prolonged Function of Porcine Islet Cell Cluster Xenografts in Macaques without Immune Suppression, in preparation, 2008.

Non Peer Reviewed Articles

1. Beschorner, W. E., and Boitnott, J. K. Liver pathology after bone marrow transplantation. Am. Soc. Clin. Pathol., Anatomic Pathology (No. APII-43), Chicago, Illinois, 1981.
2. Bender, W. L., Whelton, A., Beschorner, W. E., Darwish, M. O., Hall-Craggs, M., and Solez, K. Nonspecificity of the renal lesion of fenoprofen nephropathy. Contrib. Nephrol. 42:253-9, 1984.
3. Ringden O., Deeg, H.J., Beschorner, W., and Slavin, S. Effector cells of graft-versus-host disease, host resistance, and the graft-versus-leukemia effect: summary of a workshop on bone marrow transplantation. Transplant Proc. 19:2758-61, 1987.

Book Chapters

1. Tutschka, P.J., Hess, A.D., Beschorner, W.E., Korbiling, M., Santos, G.W. Cyclosporin A in bone marrow transplantation - Baltimore experience in preclinical studies. In *Experimental Hematology Today* (S.J. Baum, G.D. Ledney, Eds.) Springer-Verlag, New York, Inc., pp. 99-106, 1981.
2. Epstein, R.B., Bennett, B.T., Beschorner, W.E., Hess, A.D., Sarpel, S., Zander, A. Immunology and immunotherapy of the canine venereal tumor. In *Cancer Immunology: Experimental and Clinical*. (R.G. Crispin, Ed.) Elsevier/Holland, pp. 299-312, 1981.
3. Tutschka, P.J., Hess, A.D., Beschorner, W.E., Santos, G.W. Cyclosporin A in allogeneic bone marrow transplantation. Preclinical and clinical studies. In *International Symposium on Cyclosporin A* (D. J. G. White, R.Y. Calne, Eds.). Elsevier/North Holland Biomedical Press, pp. 519-38, 1982.
4. Beschorner, W.E. Localization of the cellular components of acute rejection in renal allografts. In *Kidney Transplant Rejection: Diagnosis and Treatment*. (G.M. Williams, J.F. Burdick, K. Solez, Eds.), 1986, pp 225-46.
5. Solez, K., McGraw, D.J., Beschorner, W.E., Burdick, J.F. Pathology of "acute tubular necrosis" and acute rejection. Observations on early systematic renal transplant biopsies. In *Kidney Transplant Rejection: Diagnosis and Treatment*. (G.M. Williams, J.F. Burdick, K. Solez, Eds.) 1986, pp 207-25.
6. Santos, G.W., Yeager, A.M., Saral, R., Burns, W.H., Wingard, J.R., Jones, R., Ambinder, R.F., Rowley, S.D., May, S., Vogelsang, G.B., Donnenberg, A.D., Beschorner, W.E., Wagner, J.E., and Hess, A.D. Allogeneic and autologous marrow transplants in acute non-lymphocytic leukemia (ANLL). In *Bone Marrow transplantation: Experimental Hematology Today*, (S.J. Baum, G.W.F. Santos, F. Takaku, F., Eds.) Springer-Verlag, N.Y., 1987, pp. 74-81.
7. Hess, A.D., Fischer, A.C., Vogelsang, G.B., Beschorner, W.E., and Santos, G.W. Syngeneic graft-versus-host disease: Failure of self:non-self discrimination. (S. J. Baum, G. W. Santos, and F. Takaku, Eds.) In *Bone Marrow transplantation: Experimental Hematology Today*, Springer-Verlag, N.Y., 1987, pp. 12-17.
8. Herskowitz, A., Baughman, K.L., Rose, N.R., Neumann, D.A., Beschorner, W.E., Sell, K.W., Ahmed-Ansari, A. Induction of major histocompatibility antigens on myocardial cells in patients with active myocarditis and idiopathic cardiomyopathy. In *New Concepts in Viral Heart Disease*. (HP Schultheliß, Ed.), Springer-Verlag, Berlin, Heidelberg, 1988, pp. 312-24.
9. Wagner, J.E., Yeager, A.M., and Beschorner, W.E. Pathology of Bone Marrow Transplantation. In *Bone Marrow Transplantation in Children*. (F.L. Leonard Johnson, C. Pochedly, Eds.), Raven Press, NY, 1989, pp. 141-164.
10. Beschorner, W.E. and Turnicky, R.P. Intestinal and hepatic manifestations of graft-vs-host disease. In *Immunology and immunopathology of the liver and gastrointestinal tract*. (S.R. Targan, F. Shanahan, Eds.) Igaku-Shoin, NY, p. 589-611, 1989.
11. Beschorner, W.E. Localization of the cellular components of acute rejection. In *Kidney Transplant Rejection: Diagnosis and Treatment*. (G.M. Williams, J.F. Burdick, K. Solez, Eds.), 1991, second edition.
12. Beschorner, W.E., and Lomis, T.J. Xenotransplantation: Potential Opportunity Versus Resistant Challenge. In *Kidney Transplant Rejection: Diagnosis and Treatment*. (L.C. Racusen, K. Solez, J.F. Burdick, Eds.), 1998, third edition, Marcel Dekker, pp. 201-250.
13. Beschorner, W.E. Xenotransplantation, In Maddrey W.C., Schiff E.R., and Sorrell MF., *Transplantation of the Liver*, Lippincott, Williams & Wilkins, Philadelphia, 2001, pp. 431-454.

14. Beschorner, W.E. Cardiac Xenotransplantation, In Franco K.L. and Verrier E.D. Advanced Therapy in Cardiac Surgery, B.C. Dekker, Saint Louis, 2004.

Representative Abstracts

1. Gaudin, P.B., Hruban, R.H., Kasper, E.K., Olson, J.L., Beschorner, W.E., Baughman, K.L., Hutchins, G.M. Myocarditis associated with anthracycline toxicity. *Lab Invest.* 64:18A, 1991.
2. Wojno, K.J., Beschorner, W.E., and Santos, G.W. Pulmonary hemorrhage as a cause of death in allogeneic bone marrow recipients with severe acute graft-vs-host disease. *Lab Invest.* 64:120A, 1991.
3. Herskowitz, A., Willoughby, S., Beschorner, W.E., Ansari, A.A., Rose, N.R., Neumann, D.A., and Baughman, K.L. Cardiomyopathy associated with human immunodeficiency virus (HIV) infection. Second International Symposium on Myocarditis. May 5, 1991.
4. Leffel M.S., Elkalifa, M.Y., Beschorner, W.E., and Bias, W.B. Possible role of LEWIS antibodies in renal graft failure. American Society of Transplant Physicians, May 29, 1991.
5. Zehnbaue, B., Griffin, C., Mann, R., Beschorner, W., and Ambinder, R. Cytogenetic and molecular characterization of the (11;18) translocation in lymphomas. 8th International Congress of Human Genetics. October 6, 1991.
6. Beschorner, W.E., Divic, J., Yao, X., Kenworthy, P., and Bruce, G. Recombinant human growth hormone and insulin-like growth factor I accelerate thymic recovery after cyclosporine: potential adjunct agents for the induction of tolerance. The Transplantation Society. Second Basic Sciences Symposium, Oxford, UK. Plenary session, September 5, 1991.
7. Beschorner, W.E., Divic, J., and Yao, X. Transplant tolerance develops **after** Cyclosporine. *Lab Invest.* 68:122A (#709), 1993.
8. Fox, W.M. III, Hameed, A., Hutchins, G.M., Reitz, B.A., Baumgartner, W.A., Beschorner, W.E., Hruban, R.H. Lymphocytes expressing perforin can be found in the intimas of coronary arteries with transplant related accelerated arteriosclerosis. *Lab Invest.* 68:23A (#114), 1993.
9. Beschorner, W.E., Qian, Z., Mattei, P., Hess, A.D., Colombani, P.M., Khouzami, A., Blakemore, K.J., and Burdick, J.F. Induction of human chimerism and functional suppressor cells in fetal pigs. Feasibility of surrogate tolerogenesis for xenotransplantation. Third International Congress for Xenotransplantation, 1995.
10. Lomis, T.J., Hess, A.D., Blakemore, K.J., Lynd, C., Hall, S., Paletta, J. and Beschorner, W.E. Novel Process for inducing specific immune tolerance to pig xenografts without severe immune suppression. American College of Surgeons, San Francisco, CA, October 1996.
11. Beschorner, W.E., Lomis, T.J., Hess, A.D., Dimling J., Dimling, G., Blakemore, K.J., Burdick, J.F. Prolonged acceptance of pig xenografts without severe immune deficiency. American Society of Transplant Physicians, Chicago, IL. Abstract #491, 1997.
12. Sudan, D.L., Radio, S.J., Johnson, N.D., Matamoros, A., Shearon, C.C., Fox, I.J., Herlenius G., Dixon, R.S., Langnas, A.N., and Beschorner, W.E. Prevention of hyperacute rejection of pig xenografts by surrogate tolerogenesis. American Society of Transplant Surgeons. Chicago IL, 1999.
13. Beschorner W., Sudan D., Radio S., Johnson N., Shearon C., Rubocki R., Kuszynski C, Dixon R., Matamoros Jr. A., Langnas A. Prevention of vascular xenograft rejection by induction of accommodation in the donor pig prior to transplant, Vth International Congress on Xenotransplantation, Nagoya, Japan, 1999.
14. Yang, T, Alfred Stammers, M. S., Carson Shearon, M. S., Scott Thompson, Alan N. Langnas, D. O., William E. Beschorner, M.D. Pretransplant Xenograft Accommodation. Chimeric Pig Hearts Resist Hyperacute Rejection in Langendorff Assay. American Society of Transplant Physicians, 2000.

15. Sudan D.L., Franco, K.L., Beschorner, W.E., et al. Prolonged Survival of Pig Xenografts using Chimeric Donors and Modest Immune Suppression. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
16. Shearon, C.C., Yang, T., Beschorner, W.E. et al. Peripheral Blood Lymphocytes in Chimeric Pigs Resist Lysis by Antibody and Complement. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
17. Beschorner, W.E., Shearon, C.C., Yang, T., Thompson, S.C., Langnas, A.N., Sudan, D. L., Chimeric Pig Donors producing Xenogeneic Immunoglobulin and Heart Xenograft Survival. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
18. Beschorner, W.E., Thompson, S.C., Fox, I.J., et al. Growth and Function of Human Hepatocytes in Pigs. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
19. Yang, T., Zhao, Y., Beschorner, W.E., et al. Heart Explant from Chimeric Pig (Sheep Cells) Protected from Hyperacute Rejection by Human Blood. 6th Congress of the International Xenotransplantation Association, Chicago, IL, 2001.
20. Beschorner, William E. The Advantages of Hybrid Xenografts. Third Annual Healthcare Conference, Techvest, LLC., pp. 179, 2001.
21. Beschorner, William E., Fox Ira J., and Sudan, Debra L. Chimeric pigs for xenotransplantation. Fifth International Symposium on In Utero Stem Cell Transplantation and Gene Therapy. Reno, NV, 2001.
22. Beschorner, W.E., Thompson, S.C., Yang, T., Cederberg, C., Fox, I.J., Strom, S.C. Human hepatocytes from fetal pigs infused with hepatocytes or marrow. Cell Transplant Society, 10th Anniversary Congress, 2001, p. 83.
23. Beschorner, W.E., Lechtenberg, K.F., Schieber, T., Rigley, T., Skorupa, J.Y., Wisecarver, J., Stevens, R.B. Prolonged Engraftment without Chronic Immune Suppression of Porcine Islet Tissue using Chimeric Donor Pigs. Cell Transplant Society, 14th Congress, 2005.
24. Beschorner, W.E., Lechtenberg, K.F., Schieber, T., Rigley, T., Skorupa, J.Y., Wisecarver, J., and Stevens, R.B. Prolonged function of porcine islet tissue using chimeric donor pigs without chronic immune suppression. 8th Congress, International Xenotransplantation Congress, Gothenberg, Sweden, 2005.
25. Beschorner, W.E., Lechtenberg, K.F., Schieber, T., and Dalmaso, A.P. Tissue accommodation induced in chimeric donor pigs. 8th Congress, International Xenotransplantation Congress, Gothenberg, Sweden, 2005.
26. Beschorner WE, Prather RS, Lai, L, Kudlacek P, Harbour-Marik K, Schieber T, Lechtenberg K.F., Transgenic Pigs Producing the Suicide Gene Thymidine Kinase in the Liver. Facilitation of Human Hepatocytes in the Pig Liver. International Xenotransplantation Association, 2007, Minneapolis, MN.

26. Beschorner, W.E. Hematopoietic Stem Cells and Immune Tolerance. Tolerance to Xenotransplants “Outside the Box.” International Society of Cell Transplantation. Miami, FL 2008.
27. William E. Beschorner, Patrick E. Kudlacek, Yasuhiro Takeuchi, Teresa Schieber, Kelly F. Lechtenberg. PERV Free Swine in a Defined Pathogen Free Herd. Transplantation Society, 2008.
28. William E. Beschorner, Patrick E. Kudlacek, Teresa Schieber, Kelly F. Lechtenberg. Enhancement of Human Chimerism in Donor Pigs through Inhibition of Fetal Porcine Natural Killer Cells. Transplantation Society, 2008.

Development of Human/Pig Hybrid Livers for Organ Transplantation

Ximerex, Inc. and the University of Nebraska Medical Center

There is currently a severe shortage of human organ donors. Most patients who could benefit from a human liver transplant never receive one. Those that do receive an allograft must typically wait for more than a year. Although allotransplants are usually life-saving procedures, the recipients must take anti-rejection drugs indefinitely. These drugs are often associated with complications and infections.

Alternatives to allotransplantation are being explored, including tissue engineering, differentiation of embryonal stem cells, and xenotransplantation. Each of these technologies has significant limitations preventing its use. Embryonal stem cells and tissue engineering are very promising technologies. However, major hurdles must be cleared before complex organs can be grown *in vitro*. Even optimistic promoters believe that it will be decades before that dream becomes a reality¹. There is great interest in xenotransplantation, such as pigs. Pigs can be readily produced in large numbers under clean conditions. The physiology of most porcine tissues is similar to that of humans. The risk of infection is very low.

There are two major drawbacks to the use of pigs for organ donation, however². First, the immune rejection of vascular pig organs is fulminant. In part, this is due to preformed antibodies to pigs and in part due to numerous antigen differences between the species. Second, the physiology of some organs, including the liver, is sufficiently different from humans to preclude their use.

Fulminant rejection of pig tissues cannot be overcome by conventional means, such as immune suppression. Doses normally effective in allotransplants are ineffective with the xenotransplants. Even after bone marrow transplantation, baboons fulminantly reject pig xenografts.

Genetic engineering was initially thought to hold the solution to the problem of xenotransplant rejection. Presently, however, it has only proved useful for preventing hyperacute rejection. Because of the inefficiency of conventional methods, development of useful transgenic herds has proved to be very slow.

1. Transgenic system for human/pig hybrid liver grafts

This project proposes to produce hybrid human/pig livers by culturing human liver cells within preimmune fetal pigs. The hybrid organ would provide significant advantages. Multiple species specific factors, both known and unknown would be corrected in a single step. Genetic modification of the liver would be accomplished with gene therapy applied to the human cells. This would be much easier and faster than altering the pig. For example, the cultured hepatocytes could potentially be transfected with genes conferring resistance to hepatitis viruses. The antigen disparity with the recipient would be reduced.

To be effective, the system must provide a selective advantage for the human hepatocytes to grow within the fetal pig. Otherwise the native pig cells would successfully compete with the human hepatocytes, preventing their engraftment and proliferation. Yet the native pig liver should be normal until the human hepatocytes are infused, permitting the swine to be easily bred and raised. This would be accomplished by targeting the pig cells with a suicide gene and prodrug. Suicide genes produce enzymes not normally present in the cells. The enzyme converts a non-toxic prodrug into a toxic substance. The best known system involves thymidine kinase (tk) and the prodrug ganciclovir. Tk, produced by Herpes viruses, is not normally present in uninfected cells. Herpes infected cells convert the ganciclovir and are destroyed. This system will be compared with a second system employing the gene for cytosine deaminase and the non-toxic prodrug 5-fluorocytosine. The prodrug is converted into the toxic 5FU.

To prevent significant injury to the rest of the pig, the injury will be directed to the liver. Two methods will be compared. First, immunoliposomes will be prepared containing the appropriate prodrug and carrying antibodies specific to antigens expressed on hepatocytes, such as asialoglycoprotein receptor. Immunoliposomes have been used for targeting tissues, such as delivery of cytotoxic agents to hepatomas.

Liposomes containing ganciclovir have been used to treat retinal CMV infections. The second method would construct the vectors with an albumin promoter. The suicide gene is active only within the hepatocytes.

In vitro cultures of pig and human hepatocytes will be used to compare these four methods of targeting pig hepatocytes. The pig cells are infected with the appropriate vector.

A rapid and efficient method of genetic engineering has been recently developed by R. Bremel and A. Chan³ of Gala Design, L.L.C. Genetic engineering as generally practiced, transfects an embryonal cell. This method has an efficiency of 1-3% and leads to mosaic transgenic animals, expressing the gene in select tissues. In contrast, pig oocytes would be transfected with a retroviral vector during meiosis. This method has produced transgenic cattle with efficiency close to 100%, without any mosaicism. Pilot studies indicate that the vector transfects pig cells as well.

The growth of allogeneic and xenogeneic hepatocytes is supported by several studies. Rihm and Brinster cultured mouse and rat hepatocytes in nude transgenic mice that had a functional hepatocellular deficiency (albumin urokinase deficiency)⁴. The liver was repopulated with up to 100% of the transplanted hepatocytes. Fox has transplanted normal human hepatocytes into a patient with Crigler-Najjar syndrome⁵. Engraftment significantly reduced her bilirubin and reduced the amount of phototherapy needed. Rat hepatocyte transplants have also produced notable clinical improvement in rats with cirrhosis. Recently, JR Chowdhury and I Fox have infused human hepatocytes into RAG-2 mice. A year later the livers contained lobules of 100% human hepatocytes. Overall, human cells accounted for approximately 3% of the total hepatocytes.

The development of this system would complement research done at Ximerex, Inc. and the University of Nebraska Medical Center. Funds are **not** requested here for those programs.

Although hybrid organs could be produced with hepatocytes harvested from cadaveric donors, a renewable source would provide greater predictability, and would avoid the need to screen each suspension of cells for infectious agents. Fox and Leboulch have developed a system of conditionally immortalized cells. The cells contain a transformation gene (SV40Tag) and a suicide gene (thymidine kinase) surrounded by loxP sites. The cells can be expanded indefinitely in culture. Prior to use, the cells would be infected with a vector containing Cre-recombinase. This enzyme recognizes the loxP sites and removes the transformation and suicide genes. The cells that do not revert back to a mortal state would be killed with ganciclovir. The efficacy has been demonstrated with rat hepatocytes. Dr. Fox is developing a similar human line.

The immune system may recognize the hybrid organ as an allograft rather than a xenograft and require less immune suppression than xenografts. However, it would be most useful to induce immune tolerance to both components of the hybrid organ. W. Beschoner of Ximerex, Inc. has developed a process termed "surrogate tolerogenesis⁶." Rather than attempting to induce tolerance within the patient, the patient's lymphocytes are cultured within fetal pigs. Specific tolerance is adoptively transferred back to the recipient before transplanting the pig organ. The efficacy is supported by human lymphocytes cultured in fetal pigs and pig-sheep xenotransplant models. Specific inhibition of cellular and antibody reactions to pig has been achieved. Pig xenografts are accepted by sheep without immune suppression.

The proposal is innovative, first of all, by modifying the pig xenograft through engraftment of human somatic cells rather than by genetic engineering. Multiple potential xenogeneic disparities would be corrected in a single step rather than piecemeal. Once the system is developed additional genetic alterations would be accomplished through gene therapy applied to the human cells. Genetic engineering of the pigs would be used sparingly, providing an environment favorable to select human cells.

Second, a controlled number of pig cells are destroyed through the use of tissue specific immunoliposomes containing a prodrug. This is an improvement over congenital enzyme deficiencies that affect all cells. A portion of the pig liver could be preserved. The pigs could be easily bred and raised. They would not be compromised until exposed to the prodrug.

Third, the proposal is innovative in that it involves the novel combination of innovative processes backed by patents submitted by the participants or issued to the participants. Bremel and Chan have received a patent

for retroviral transfection of oocytes. Beschorner has applied for a patent for surrogate tolerogenesis (issued in Australia, allowed in the United States, pending in Canada, Europe, and Japan). The patent includes claims for the engraftment of pig organs with human somatic cells. Fox and Leboulch have a patent pending for conditionally immortalized hepatocytes.

Though the system will be used initially with human hepatocytes to produce hybrid livers, it could readily be adopted to other somatic cells, including progenitor cells derived from embryonal stem cells, endothelial cells, smooth muscle cells, and neural stem cells. This would be most easily accomplished with the immunoliposome system, employing antibodies specific for the targeted cells. If the tissue specific promoter is employed, new transgenic pigs would be necessary.

As expected with any unproven technology, this proposal would be considered high risk. The project plan, however, is designed to reduce the risk and significantly enhance the likelihood of success. Individual components are supported by preliminary studies. Feasible alternatives are planned. The research and development plan places the greatest risk at the beginning of the project, allowing for a change in procedures earlier rather than later.

2. Development

Development of new technology

In the initial phase, vectors and immunoliposomes (ilsm) would be developed. The vectors would contain the suicide gene and albumin (alb) promoter if appropriate. The immunoliposomes would contain the prodrug and tissue specific antibodies. *In vitro* cultures would assess the specificity of the four systems (tk/ilsm, cd/ilsm, tk-alb, cd-alb) to kill the pig hepatocytes with minimal injury to human hepatocytes. The specificity of liver injury and human hepatocyte engraftment would also be tested in mice and the systems compared.

Transgenic pigs would then be produced containing the appropriate suicide gene and promoter. The piglets would be screened and transfected pigs crossbred to produce homozygous animals.

Assessment

With the development of second generation transgenic pigs, preimmune fetal pigs would be injected with the immunoliposomes or prodrug and human hepatocytes. The chimerism would be assessed short term and long term. Appropriate studies would assess the production of human complement and other factors.

The system will be assessed with orthotopic transplants of hybrid livers into non-human primates. Liver chemistries, chimerism, and clinical status would be monitored for a prolonged period.

These studies would provide support for human clinical trials. Funding is not requested for clinical trials.

Initial clinical trials would use hybrid human/pig livers as a temporary bridge transplant for patients in acute liver failure. They would be done in compliance with FDA guidelines. The University of Nebraska Medical Center has performed 14 extracorporeal liver perfusions with unmodified pig livers. If justified, the studies would lead to clinical trials with long term hybrid liver transplants. The UNMC is recognized for its excellent liver transplant program, performing more than 100 transplants per year.

3. Benefits to the US economy

There is a severe shortage of human organ donors. Approximately 5000 top priority patients are waiting for a liver transplant at this time. The procedure is highly effective, with greater than 90% survival at 3 years. The procedures cost approximately \$110,000 each. The actual number of patients that could benefit would be much greater, estimated to be 52,000 per year world-wide (25,000 in the U.S.).

The technology could be adopted for other tissues as well. For example, kidneys and hearts could potentially be produced with the vasculature lined with human endothelial cells. Because the liver produces

hematopoietic growth factors that are species specific, such as stem cell factor, the engraftment of human hepatocytes could enhance hematopoietic and lymphocyte chimerism, enhancing tolerance to other tissues. Financial projections have estimated that as many as 455,000 organs could be transplanted per year, if the technology and organs were available⁷.

4. Business plan

Ximerex, Inc. would serve as a tissue bank, providing hybrid organs to transplant surgeons. The Company would also induce tolerance to the organs for specific patients. A sample of marrow would be received and infused into the fetal pigs along with the liver cells and immunoliposomes. The resulting chimeric pigs would be screened for chimerism and tolerant lymphocytes. A kit containing the tolerant lymphocytes and hybrid liver would be sent to the hospital performing the transplant.

The technology of genetic engineering using retroviral infection of oocytes would be licensed from Gala Design, L.L.C. The development of vectors and training of personnel would be subcontracted to them.

Procurement of human organs for transplantation currently costs about \$20,000 each. The high cost is due to several factors, including the need to maintain the donor on life support, the need to harvest the organs on an emergency basis, tissue typing, etc. Hybrid organs and tolerant lymphocytes could be provided at a similar rate, including a 40% profit margin. Since these organs would require less immune suppression than other technologies, it would compete well. Potential revenues would amount to \$1 billion for liver transplants and \$9 billion for all vascular organ transplants.

5. Why are ATP funds necessary?

For the past several years, investment funds for small biotechnology companies have been very limited. Start-up companies are considered a poor risk because their success is dependent on a single technology without clinical evidence of efficacy. This is a novel and untested approach in a new field.

Ximerex, Inc. is a start-up biotechnology company. It is devoting current funds to development of surrogate tolerogenesis for heart and kidney transplants. The proposed R&D for producing human/pig hybrid livers would be too costly.

The proposed project would broaden the technology base of the Company, providing a second transplant technology for Ximerex, Inc. The proposed R&D would support an IND application for clinical trials. These two developments would enhance the ability to obtain investment funds.

References:

- ¹ Mooney DJ and Mikos AG. Growing New Organs. *Scientific American*. 280:60-65, 1999.
- ² Beschorner WE and Lomis TJ. Xenotransplantation: Potential Opportunity Vs Resistant Challenge. In *Kidney Transplant Rejection*, LC Racusen, K Solez, JF Burdick, eds, M. Dekker, NY, pp.201-250, 1998.
- ³ Chan AWS, Homan EJ, Ballou LU, Burns JC, Bremel RD. Transgenic cattle produced by reverse-transcribed gene transfer. *Proc. Natl. Acad. Sci.* 95:14028-33, 1998.
- ⁴ Rhim JA, Sandgren EP, Palmiter RD, Brinster RL. Complete reconstitution of mouse liver with xenogeneic hepatocytes. *Proc. Natl. Acad. Sci.* 92:4942-6, 1995.
- ⁵ Fox IJ, Chowdhury JR, Kaufman SS, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 338:1422-6, 1998.
- ⁶ Lomis TJ, Hess AD, Blakemore KJ, Lynd C, Hall S, Paletta J, Beschorner WE. Novel Process for inducing specific immune tolerance to pig xenografts without severe suppression. *Surg. Forum* 47:411-3, 1996.

⁷ Laing P. The Unrecognized Potential of Xenotransplantation. Salomon Brothers/Sandoz, 1996.

PROJECT Höechst / Hepatocyte Prep.
 3/11/99
Höechst medium

Notebook No. _____

37

Continued From Page _____

Isocoves MDM \bar{c} L-glutamine \bar{c} 25 mM HEPES buffer } GIBCO BRL cat. #12440

+ 17% penicillin + streptomycin } GIBCO BRL #12146-122
 + 2% FBS (heat inactivated) } GIBCO BRL cat. #16250-050

3/17/99

HEPATOCTE PREPARATION

IRA Fox - office ext. 9-8859 pager: 2385

obtain cells from Cell Processing ext. 97626

Jan, Donna, or Amy

* allow approx 30 mins. for cell preparation.

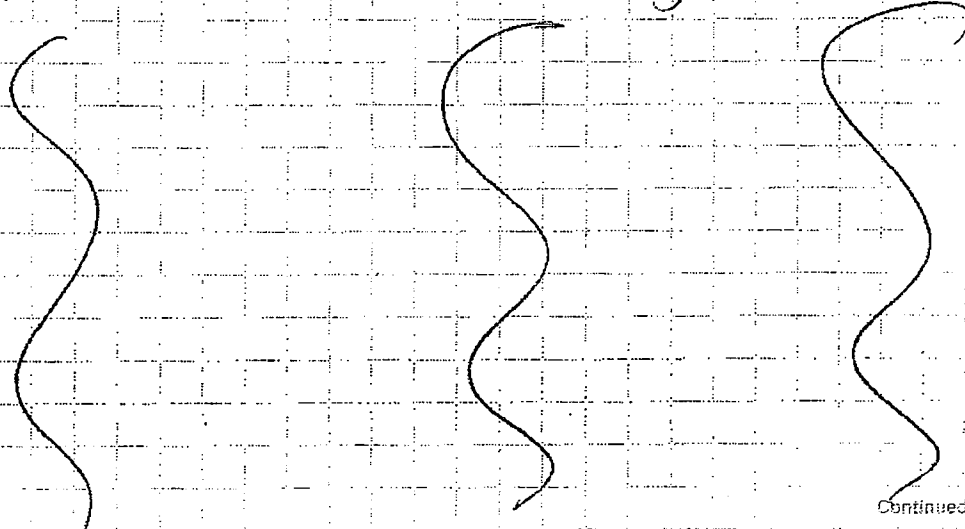
- cells will be in UW medium.

- spin @ 35 G (420-450 rpm) 4°C for 10 mins.

- wash in lactated ringers soln. (x2)

- bring cells up in fact. ring. soln.

- place 15 million cells in 1,350 μ LRS in round bottomed push cap tube (keep on ice until injection).



Continued on Page _____

Read and Understood By _____

OC Shearer

Signed

3/17/99

Date

Signed

Date

189

Injection of human hepatocytes 3/17/99
into fetal p.g.s.

15×10^6 human hepatocytes obtained from I For.
Contr. taped @ 400 RPM. Resuspended in lactated
Ringers at $1.2 \times 10^7/\mu\text{L}$ ($3 \times 10^6/0.25 \mu\text{L}$).

Sow 75 anesthetized @ ketamine + xylazine.
Masked @ isoflurane to effect, CR = 85 mm.
Abdomen prepped. 6 p.g.s injected = $0.2 \mu\text{L}$ (12.4×10^6)
each, injected into livers.
Pigs doing well afterwards.

3/19/99

All 4 sows from 3/12/99 aborted their fetuses -
See Noel's notebook

1, 87, 98 on 3/15/99 (3 days)

35 on 3/18/99 (6 days)

Histology obtained on most fetuses.

W E Basch 3/19/99

475 (12/11/10)

475 (12/21/98)

5.9

5.9

Histology from sheep 477

201

Chimeric (17) 84-5 p.s. ~~stop~~ spleen cells
and heart graft into multiply sensitized
sheep.

5/12/99

Heart graft showed pure hyperacute
rejection - Extensive interstitial hemorrhage,
arterioles & platelet plugs scattered
neutrophils. No interstitial infiltrates
to speak of.

Euthanized & autopsied sheep 3

5/19/99

Sow #75 ♀ (human hepatocytes, p189) farrowed
spontaneously. However, there were 5 full term stillborns,
1 mummified fetus and 3 live births. Live births
suckling & doing well. Stillbirths put into ref. g.

5/22/99

Still births autopsied. Gender noted. Sections of liver, spleen,
tongue, skin, gut for histology. Sections of liver & spleen frozen,
tissue preps for FISH, cell suspensions for FISH.

5/24/99

Reviewed histology from sheep 3

5/26/99

Heart graft showed neutrophils in heart graft.
Myofibers were intact. Lymphocytes rare -
May have been a resolving rejection. Lymphs were
sensitive to steroids & CSA.

W E Foulness 5/26/99

5.9

202

Noel + Carson authorized 5/26/99
 3 live pigs from Sow 75 (hepatocytes)
 #4 - the liver appeared more yellow than others.
 (p.g. was female) - otherwise, no real difference.

5/26/99
 T. Yang did laparotomy & A Stamens.
 2 pigs. First p.g. forgot to heparinize p.g.
 Went nowhere.
 Second p.g. heart beat for ~ 15 minutes.
 Problem: looking. T. Yang - correcting problem.

5/27/99
 Sow 75 authorized. No more p.g.s. in uterus.

5/28/99
 T. Yang made adjustments to laparotomy apparatus. Smaller tube & heater for apparatus providing pressure. Cut off tip of ext tube for bubble chamber holding heart, improving perfusion. Heart beat for over an hour when it was discontinued.

5/28/99
 Noel did PCR for human vs. pig DNA
 Detectable at 1:1000, not detectable at 1:10,000. Not sure if nested or primary.
 over
 reject.

W. Becker 5/28/99

Human hepatocyte transfer

6/9/99

Slides reviewed from piglets for sow #95
(human hepatocyte injections)

Piglet

75-1

#6, 7, 8 piglets were born live & euthanized at 4 days.

75-2

Liver - difficult to identify 2 populations of hepatocytes. No nodules really apparent.

In 7 and 8, apparent needle tracks seen.

75-3

In #8, most of the hepatocytes had fat accumulation. One region, however, showed very little fat. Not sure if this is a second population of hepatocytes or just variability within the pig cells.

75-4

Spleen reviewed. All three spleens showed numerous large pale pink cells, many with prominent nucleoli. Could not make out definite liver structure. One possible ductule seen. Increased extramedullary hematopoiesis present, compared to normal pig control (3-6).

75-5

PCR - Prominent bands seen in 5 of the 7 pig spleens tested. Human hepatocyte +, Pig control (-), no bands.

The most prominent bands were of the same molecular weight as the human control. Faint bands seen corresponding to human controls (500 bp) in 4 spleens - 2 of the stillborn, 2 of 3 live births (#6, 7).

75-7

Will redo #8. Each of the 5 had a prominent band, similar to control, but higher MW ~1000 bp. Polymorphisms? Dimerization? W. S. B. 6/9/99

lap
hep
EM
ofMu
lap
hep
Same
control

Histology Hepatocyte Injection in premature fetal pigs

205

Site	Spleen	Liver	6/10/99	
5-1	Lymphs + stroma No apparent hepatocytes	Autolysis (severe)	Autolysis Aerated	
5-2	Many nodules of pink large cells	Autolysis Interstitium hemorrhage	Aerated, capsted	13
5-3	Numerous nodules large pink cells c/w hepatocytes No EMH	Autolysis Interstitium hemorrhage/ congestion	Aerated, alveoli open capsted	n) g
5-4	Lymphs, stromal cells	Autolysis (severe)	Alveoli collapsed	3 6
5-5	Stromal cells (lymphocytes)	Autolysis (severe)	Alveoli collapsed	
5-6	Numerous nodules of large pink cells c/w hepatocytes. Increased EMH. Some suggestion of cords + early b. l. ducts	Apparent necrosis + thickened lobule near by had hepatocytes minimal fat. Else where, hepatocytes had mod fat accumulation Apparent central vein hepatocytes + RBC	Not seen	
7	Numerous nodules large pink cells c/w hepatocytes. Increased EMH Some suggestion of cords + b. l. ducts	Mild moderate distortion of lobules with decrease of central veins WFB	Aerated, alveoli open	5.9

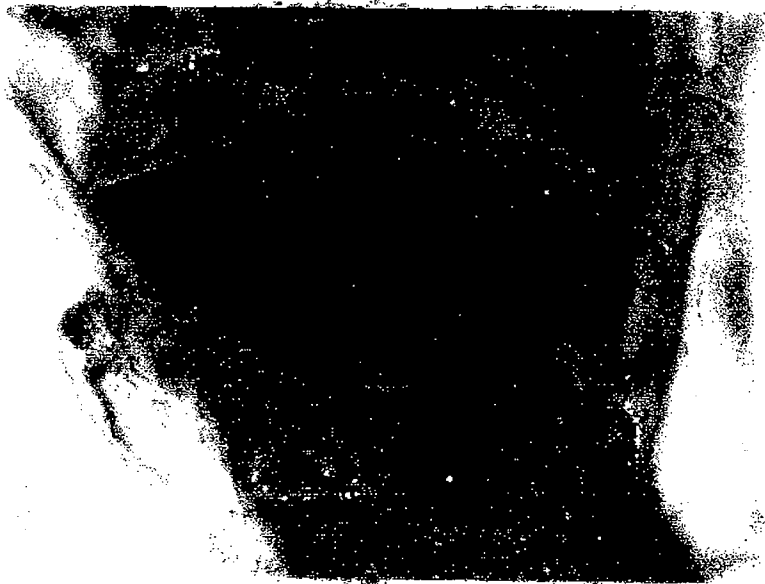
6/10/99

206
75-8

Spleen
Numerous nodules
in Red Pulp of Spleen
pink cells c/w hyper-
plasia. Inc.
Em H. Suggestion
of cords.

Liver
Needle Tract
apparent.
Liver seems
homogeneous

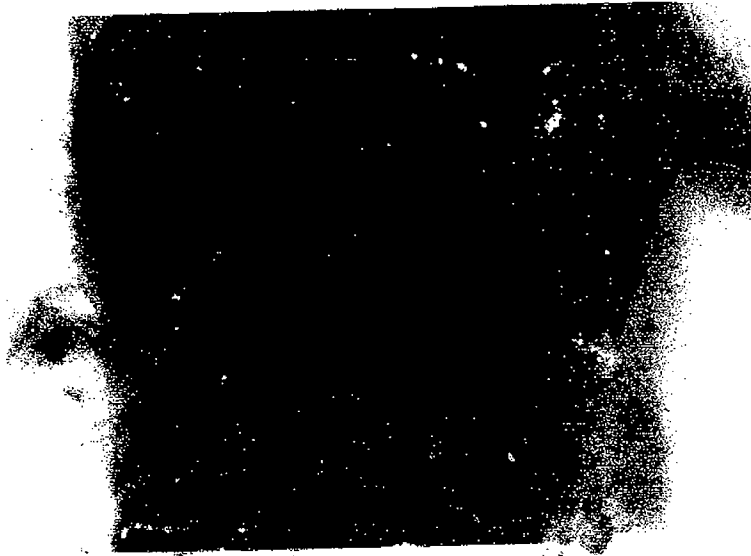
Lungs
Muc, alveoli open
aerated



75.6

Note areas of liver are dark or
light. Could represent hepatocellular changes

6/13/99 W22 Becker



III

41

m

7.

6

4

4.

5.

4.

4.

4.

5.

5.

5.

4.

4.



7-8

Some areas appear hyperemic

6/13/98 WJ Beal

210

Hybrid Liver

Noel reported that he did PCR on 6/13/99
p.s. from cow 95 - Splenocytes, using second
set of primers. Demonstrated bands on the
five p.s. spleens at appropriate MW for human
Pig DNA Negative. Good confirmation of hepatocellular
chimerism.

6/15/99 W. J. Beck

70

PROJECT _____

Notebook No. _____

Continued From Page _____

Sheep 3 euthanized 5/13/99

graft heart measured 6 x 4.5 cm
 Left Ventricle 1.2 cm cross-section
 RV 1.0 cm

Very difficult to distinguish anatomical detail. all ~~surface~~ surfaces had adhesions to surrounding sheep tissue (didn't look like fibrous though). Chambers were solid w/ clotted blood. Portions of heart wall were black - same color as clot.

5/24/99 necropsies on litter 75
 stillborn pigs (born 5/22-23)

piglet 1 male - all organs ~ same color
 2 female
 3 male
 4 male - all organs - yellow stained
 5 female

made cell suspensions of liver + spleen
 in 5% DMSO + RPMI

touch preps of liver + spleen

OCT blocks of L+S
 histology formalin fixed cassettes of
 GVH organs + Liver + spleen blocks
 remaining livers + spleens frozen

Continued on Page

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5/26/99

offer 25 live births - euthanized
+ posted fresh

6	male	3.8	16s
---	------	-----	-----

7 male, 4.9

8 female 3.5 ²⁵⁰ - yellow liver

2 additional unarmored fetuses
passed from this litter
- no samples taken

5/28/99

PCR

Sensitivity of Human Primers: $\begin{cases} \text{CH46} \\ \text{B2 DR} \end{cases}$

Human DNA from whole blood			
<u>diluted</u>	100%	1	
in TE	1:10	2	

1:100 3

1:1000 4

1: 10 de 12 5

1. 100.000

11,700,000	6
11,700,000	17

pig DNA

Tag mit

Samples 1-8 run w/ both primer pairs

Continued on Page:

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Date _____

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PROJECT _____

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Litter 175

DPA
DFB

175-1

175-2

175-3

175-4

175-5

175-6

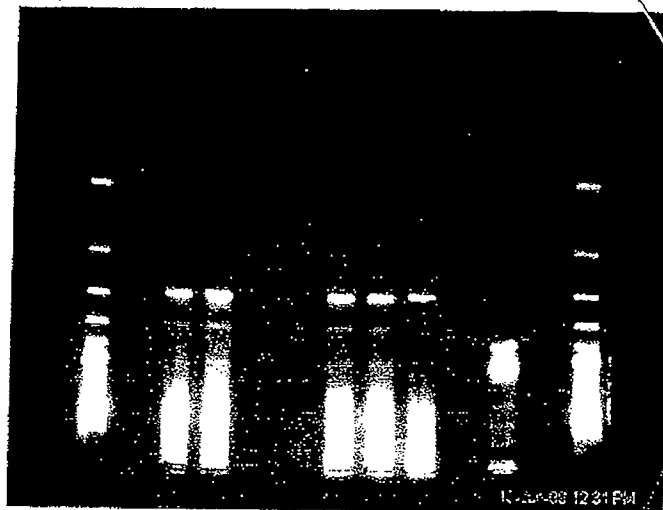
175-7

175-8

Pig C

Human Control

Neg. Mix



All DNA from Spleens
except Pig control (blood)
and Human Liver pos control

Pig Primers

- samples 1, 4, 5

have low yield
from DNA isolation



Continued on page _____

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Date _____

Confidential Information

Beschoner, William E.

RESEARCH PLAN

A. Specific Aims

There is currently a severe shortage of human organ donors. Most patients who could benefit from a human liver transplant never receive one. Approximately 4,000 liver transplants are performed annually in the United States. Those that do receive a human allograft must typically wait for more than a year. As of March 31, 1999, 12,600 high priority candidates are waiting for a liver transplant. Because of the stringent criteria for the list, the actual number of patients who could benefit from transplant is far greater. It has been estimated that 450,000 organ transplants could be performed annually, including 52,000 liver transplants, if the organs were available.

This project proposes to develop pig liver xenografts repopulated with human hepatocytes. The system will consist of transgenic fetal pigs expressing a suicide gene in the hepatocytes, the corresponding prodrug, and a method for infusion of human hepatocytes into preimmune fetal pigs. An appropriate dose of prodrug will lead to limited injury of the fetal liver. Shortly thereafter, the human hepatocytes will be infused into the fetal pigs, repopulating the injured liver. The reconstituted human/pig hybrid liver would be more physiological than a pig liver xenograft.

The proposed system depends on two underlying hypotheses. First, it is postulated that human hepatocytes are sufficiently similar to pig hepatocytes to survive and proliferate in fetal pigs. Second, the primary barrier to repopulation of the liver with hepatocytes is believed to be competition with the native pig hepatocytes. Preliminary supporting data is presented below.

In phase I of the plan, two issues critical to the success of the program will be addressed, the culture and function of human hepatocytes in pigs and the development of transgenic pigs expressing a suicide gene such as thymidine kinase in the liver. Phase II will develop an effective system, including optimizing the prodrug, timing, and hepatocyte injections and develop boars with the transgene. Phase III will include preclinical transplant studies in non-human primates with subsequent clinical trials.

Aims for Phase I

1. Determine the survival, proliferation, and function of human hepatocytes infused into preimmune normal fetal pigs. Following the birth of the pigs, the serum will be monitored for up to one month of age for human liver proteins such as albumin, alpha-1 antitrypsin, and serum amyloid A. Tissue sections will be assessed by polymerase chain reaction and fluorescent in situ hybridization studies for human cells. It is anticipated that there will be limited repopulation of the liver in the non-transgenic pigs. The hepatocytes would be expected to be present in other tissues, such as the spleen. The production of human liver proteins greater than 0.5% of the human levels would indicate that the human hepatocytes not only survived, but significantly proliferated in the pig and provided some function.

2. Using a system of retroviral transfection of pig oocytes, develop transgenic pig embryos including transgenes for thymidine kinase under the albumin promoter. This novel approach provides for nearly 100% efficiency and avoids the development of mosaics¹. The vector will be constructed and transfected into oocytes. Oocytes or zygotes are implanted into sows. Fetal pigs are assessed for the marker gene.

8/14/99 SBIR Grant

3. Assess the relative sensitivity of hepatocytes in implanted transgenic fetal pigs to the prodrug ganciclovir. The prodrug, ganciclovir, will be administered to foster sows implanted with the transgenic pigs. Seven days later, the fetal pigs will be assessed and the relative injury of the liver compared with controls not receiving prodrug and with other tissues. Because standard methods of genetic engineering are relatively inefficient and produce mosaics, considerable breeding and cross breeding is necessary before the studies of feasibility can be performed. With large animals, that may require several years. Feasibility studies with transgenic rodent models and gene therapy help but may not translate. With the high efficiency offered by transfection of oocytes, founder pigs can be tested directly to prove the feasibility. If the desired effect is seen, then other transgenic animals can be produced and the results confirmed with the herd. This advantage allows us experience failure early rather than late.

B. Significance

Competing Technologies and Justification for Human/Pig Liver Xenografts

Alternatives to the transplantation of human liver allografts are being explored, including hepatocyte transplants, tissue engineering with differentiation of embryonal stem cells, artificial organs, and xenotransplantation. Each of these technologies has significant limitations.

Instead of transplanting whole organs, one field of research would transplant a suspension of cells that would repopulate a diseased organ. For example, recently the University of Nebraska Medical Center performed the first clinical hepatocyte transplant into a patient with Crigler-Najjar Syndrome, with a congenital deficiency of a liver enzyme necessary for the conjugation of bilirubin². The patient experienced significant clinical improvement including decreased bilirubin levels. Approximately 5% of the native liver was replaced by the transplanted hepatocytes.

While hepatocyte transplants have considerable potential for correcting metabolic disorders, they are probably of little benefit for many liver diseases, such as malignancy, bile duct disorders, and portal hypertension with cirrhosis. The transplanted hepatocytes would also be susceptible to viral infections, providing minimal benefit to patients with fulminant hepatitis.

Although hepatocyte transplants can use some donated livers that are not suitable for transplant, if practiced on a wide scale, they would compete for the limited pool of human organs.

The recent development of human embryonal stem cell lines, capable of indefinite self replication and pluripotential differentiation, has generated considerable excitement and sparked speculation that human organs might be grown *in vitro*. The potential advantages of this feat are obvious. Unlimited numbers of human organs could be produced and provided as needed. Furthermore, using nuclear transfer, the genome of the recipient could be theoretically inserted into the ES cells. The resulting organ should then be antigenically identical with the recipient.

Some success has already been achieved with simple structural tissues, such as cartilage and skin. The *in vitro* production of complex organs, such as hearts, kidneys, and livers, is much more challenging. For example, ES cells can readily be induced to differentiate into rhythmically beating myofibers, the basic building block of the heart. The ontogeny of the four chamber heart, however, is a complex process influenced by the timely expression of local growth factors, the mechanical effects of adjacent tissues, the appropriate blood flow, and the migration of other cell types such as mesothelial cells. In the fetus, the local environment changes continuously. Minor

The development of the proposed technology is supported by pending and allowed patents. A patent for surrogate tolerogenesis has been issued in Australia, has been allowed in the United States and is pending in Europe, Canada, and Japan. The patents include claims for repopulating the liver in part with somatic cells, including human hepatocytes. Dr. Fox and Leblouche have filed a patent for the composition and method of producing conditionally immortalized hepatocytes. Dr. Bremel has patents pending for the retroviral transfection of oocytes.

Preliminary Supporting Data

Proliferation and function of human hepatocytes in fetal pigs. Human hepatocytes provided by S.C. Strom were infused into preimmune fetal pigs (45 days gestation) by percutaneous injection using ultrasound guidance. Two million hepatocytes were infused into each of six fetal pigs. This would be approximately 1% of the fetal pig hepatocytes. The sow came to term and delivered three live births and 5 fully formed stillbirths. The 3 live pigs were euthanized at four days of age. All of the pigs were of normal weight and showed no significant pathology. Polymerase chain reaction assays were performed using primers for HLA class I antigen. Human DNA was demonstrated in the spleens of all three live births and two of the stillbirths. Initial studies of the liver were negative. Histology of the spleens showed numerous apparent hepatocytes, constituting 10 to 30% of the section. Fluorescence In Situ Hybridization to identify human cells tissue sections are pending.

Serum was analyzed from the three live pigs by Western blot analysis for human alpha-1 antitrypsin (AAT) and ELISA for human serum amyloid A (SAA) protein. The results were compared with normal human and pig sera. Normal swine serum was negative. The chimeric pigs, however, demonstrated 0.9%, 5.5% and 23% of the levels of AAT present in the human. The SAA levels were 0.1%, 0.12%, and 0.23% of the levels seen in normal humans. The discrepancies may reflect decreased SAA production in newborns. Assuming that the serum protein is proportional to the number of hepatocytes, the number of human hepatocytes would be 10 to 230 times the number injected. The pigs grew by a factor of 100 since the injection.

The preliminary findings, therefore, suggests that human hepatocytes replicate appropriately and produce human proteins within fetal and newborn pigs. The environment within the fetal pigs, including hepatocellular growth factor, support the growth of the human hepatocytes. In contrast to the experience in mice, the porcine growth factors supported the prompt proliferation of the human hepatocytes. Furthermore, the localization to the spleen and limited engraftment in the liver is consistent with the belief that the infused hepatocytes are competing with native hepatocytes.

In a second sow, 8×10^6 human hepatocytes (approximately 4% of native hepatocytes) were infused into each of two fetal pigs and 2.6×10^6 hepatocytes infused into each of two additional fetal pigs. As of three weeks, the pigs appear healthy by ultrasound.

The technology proposed here will be combined with surrogate tolerogenesis (ST) to minimize the need for immune suppression. That technology was developed, in part, with another SBIR proposal, DK50737. Tolerance is induced to the pig, within the pig and transferred back to the recipient. With human marrow, high titers of antigen specific regulatory cells were produced which inhibited the MLR²⁴. Transplanting pig aortas into sheep, ST led to average graft survivals of 77 days (vs. 7 days control)⁵. No post-transplant suppression was given. With pig heart to sheep transplants, vascular rejection was prevented, though the sheep did mount a cellular

Beschorner, W.E. Proprietary Information 03/07/00
Human/Pig Hybrid Livers for Transplantation

performed the first clinical hepatocyte transplant, leading to a dramatic clinical improvement in a patient with Crigler-Najjar syndrome³⁴.

The personnel at Gala Design, LLC, will facilitate the development of the transgenic pigs expressing the appropriate suicide gene. They developed a highly efficient method for producing transgenic animals, using a retroviral transfection of oocytes⁸. Their technology received a very favorable review in Science³⁵. A patent is pending. These methods will be adopted to pigs by Matthew Wheeler at the University of Illinois. He has extensive experience producing transgenic pigs for agricultural and transplantation^{36, 37}.

Supporting Preliminary Studies

The following preliminary studies support the feasibility of the proposal and enhance the probability that the goal of repopulating the pig livers with human hepatocytes will be achieved. Human hepatocytes were infused into preimmune fetal pigs. The secretion of human liver proteins and the large number of hepatocytes in the spleen demonstrate that the environment in the pig supports the long-term survival of human hepatocytes. The relative paucity of human hepatocytes in the pig liver supports the basic premise of the proposal that "space" must be created through the controlled injury of native hepatocytes to achieve significant engraftment. The transfection of porcine renal tubular epithelial cells by reverse-transcribed gene transfer supports the feasibility of this highly efficient method in pigs. The experiments with surrogate tolerogenesis demonstrate that rejection of pig xenografts can be prevented without the need for severe immune suppression. The studies also demonstrate our experience with engraftment and growth of xenogeneic cells in preimmune fetal pigs.

Proliferation and function of human hepatocytes in fetal pigs.

Human hepatocytes provided by S.C. Strom were infused into 11 preimmune fetal pigs (45 days gestation) in three sows by percutaneous injection using ultrasound guidance. Two to 10 million hepatocytes were infused into each fetal pigs. The sows came to term without aborting any fetuses. Two sows farrowed naturally and one was delivered by Caesarian section.

The first sow produced three live births and 5 fully formed stillbirths. The 3 live pigs were euthanized at four days of age. All of the pigs were of normal weight and showed no significant pathology. Polymerase chain reaction assays were performed using primers for HLA class I antigen. Human DNA was clearly present in the spleens of all three live births and two of the stillbirths. PCR studies of multiple biopsies of the livers were negative. Histology of the spleens showed numerous contiguous hepatocytes, constituting 12 to 30% of the section. The number of hepatocytes was estimated from the volume of the spleen, the calculated volume of the hepatocytes and the average hepatocyte volume.

Serum specimens from the pigs were analyzed by Western blot analysis for human alpha-1 antitrypsin (AAT) and by ELISA for human serum amyloid A (SAA) protein. The results were compared with normal human and pig sera. Normal swine serum was negative.

3/7/00 ATP Grant

Proliferation of Human Hepatocytes in Pig Spleens And Detection of Human Liver Proteins

<i>Piglet</i>	<i>Hepatocytes in the Spleen</i>	<i>Est. Expansion of Injected Hepatocytes*</i>	<i>Human Serum amyloid A#</i>	<i>Human Serum AAT#</i>
75-1	3.6x10 ⁷	18X	0.12%	0.9%
75-2	1.6x10 ⁸	90X	0.23%	22%
75-3	5.8x10 ⁷	29X	0.10%	5.5%
75-4	7.6x10 ⁷	38X	ND	ND
75-5	1.6x10 ⁸	80X	ND	ND
316-1	ND	--	0.90%	ND
316-2	ND	--	0.73%	ND
316-3	ND	--	0.24%	ND
361-1	ND	--	0.45%	ND
361-2	ND	--	0.52%	ND

*Number of splenic hepatocytes/Number of Injected Hepatocytes

Concentrations compared with concentration in human control sera.

The second and third sows produced six and eight live births. Serum analysis for SAA and alpha-1 antitrypsin were consistent with chimerism in two and three pigs respectively. These pigs have been followed for up to three months. At three months one of 2 pigs (316-2) shows persistent human proteins (0.6% SAA). That pig currently weighs 20 kg and is clinically normal and healthy. At 60 days of age, three of 4 evaluated pigs had human SAA in the serum.

The preliminary findings, therefore, support the proliferation and function of human hepatocytes in the fetal and newborn pigs. The injected hepatocytes expanded at least 18 to 90 fold and produced human proteins. The detection of human liver proteins in the serum confirms the presence of viable hepatocytes in the pigs. Furthermore, the localization to the spleen and the failure to identify hepatocytes in the liver is consistent with the hypothesis that the infused hepatocytes are competing with native hepatocytes.

Transfection of Porcine Renal Epithelial Cells.

By introducing a retroviral vector into oocytes rather than targeting embryos, Chan and Bremel were able to achieve a very high efficiency of transfection and avoid the problems of mosaicism⁸. The transfections were performed with bovine oocytes.

To determine the feasibility of this technology with porcine cells, a cell line of porcine renal tubular epithelial cells (PK-15) was utilized. The vector was a replication defective vector based on the Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus. The vector included a neomycin phosphotransferase and a reporter gene (GFP, green fluorescent protein). The PK-15 cells were transfected with a high titer of the vector and the resistant cells selected. The cells were cultured for three weeks. They demonstrated diffuse expression of the GFP.

Prevention of Xenograft Rejection with Surrogate Tolerogenesis.

As an alternative to inducing immune unresponsiveness *within the recipient*, we propose instead to induce immune tolerance to the donor *within the xenograft donor*, and adoptively transfer the tolerance back to the recipient. We have termed this process "surrogate tolerogenesis"³⁸. The induction of tolerance within the donor animal provides considerably greater flexibility and opportunity. For example, with current approaches developmental tolerance could only be applied to fetal or newborn patients. With surrogate tolerogenesis, however, developmental tolerance can be established within fetal donor animals

PROJECT _____

Notebook No. _____

Continued From Page _____

1/11/00

Meeting w/ Bill -

I discussed my reluctance to use the CMV promoter, suggested alternatives such as AFP/Alb driving GFP + suicide gene - then crossing w/ GFP driven by leukocyte specific promoter

He wants to keep CMV

→ Constructs:

rTTA	CMV	GFP	IRES	ATK	IRES	YCD
rTTA	CMV	GFP	AFP	DTK		
rTTA	CMV	YFP	Alb	YCD		

FCU/Kur fusion?

Alb YFP IRES YCD

AFP GFP IRES DTK

if no rTTA

We also decided I should look for a ~~constitutive~~ ^{inducible} ~~constitutive~~ ^{inducible} promoter

Continued on Page _____

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Date _____

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Date _____

Beschorner, W.E. Proprietary Information 04/13/99
Development of Human/Pig Hybrid Livers for Organ Transplantation

7. **Production of homozygous transgenic boars, third year.** These would not be produced until the end of the project and therefore would not be useful for the scientific studies. They will be necessary for clinical and commercial application.
8. **Establish specificity of hepatocyte depletion in transgenic fetal pigs, third year.** These are short-term studies defining the effectiveness and specificity of the hepatocyte depletion.
9. **Demonstrate enhanced engraftment of human hepatocytes in transgenic fetal pigs, third year.** The level of engraftment in newborn pigs is assessed by fluorescence in situ hybridization (FISH), flow cytometry, and quantitative PCR. Human liver proteins are also measured in the serum. The levels are compared with the baseline established in milestone 2. The goal is to achieve 30 to 80% human hepatocytes. Livers with this level of human hepatocytes should be functional in human recipients. The system could also be used as a bioreactor for expanding human hepatocytes for direct hepatocyte transplants.
10. **Assess long term efficacy and toxicity in chimeric transgenic pigs, third year.** Percutaneous liver biopsies and serum are sampled on a monthly basis. The relative hepatocellular chimerism and levels of human liver proteins in the serum are determined as above. At the end of three months, the pigs are necropsied and examined for possible toxicity, tumors, infection, etc.

Specific plan for first year

Development of Transgene Constructs and Vectors

One set of constructs will contain a liver-specific promoter such as the promoter for albumin or liver-enriched activator protein (LAP; Kistner et al., 1996) fused to the cDNA encoding the suicide gene (tk or cd) and a marker gene, green fluorescent protein (GFP). The liver-specific promoter and suicide gene (tk or cd) will be subcloned into a commercially available GFP expression vector (EGFP-1; Clontech, Palo Alto, CA). Another set of constructs will contain a ubiquitous promoter such as cytomegalovirus (CMV) promoter fused to the cDNA encoding the suicide gene (tk or cd) and GFP. The suicide gene (tk or cd) will be subcloned into a GFP expression vector (EGFP-N; Clontech) which already is driven by the CMV promoter. Bacteria will be transformed with complete vectors, grown up, and DNA will be prepped, digested and run on an agarose gel for confirmation.

The complete vectors will then be subcontracted to Gala Design LLC for construction of retroviral vectors. The constructs will be inserted into replication-defective vectors based on Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus (VSV-G). In contrast to the native retroviral envelope proteins, absent from this vector, the VSV-G interacts with phospholipid components of the host cell plasma membrane. High titers of the particles (10^9) will be produced and provided to Ximerex, Inc.

Development of transgenic pigs

***In Vitro* Maturation (IVM)**

Ovaries (200/week) will be obtained from a local abattoir and transported in saline at 39°C. Oocytes will be aspirated from follicles 2-5 mm in diameter and selected for quality prior to maturation. Oocytes (n=50) will be cultured in drops of maturation medium under oil at 37°C in a 5% CO₂ in air environment for 20 hours in presence of hormonal supplements and another 20 hours in the absence of hormonal supplements. Following 40-44 hours of culture, oocytes will be stripped of

From 4/13/99 ATP, rand Page 24

Beschoner, W.E. Proprietary Information 03/07/00
Human/Pig Hybrid Livers for Transplantation

To accomplish these goals, we propose to develop a system consisting of transgenic pigs containing a suicide gene, a prodrug that would specifically destroy most of the pig hepatocytes, and infusion of human hepatocytes into the preimmune fetal pig along with the prodrug or immunoliposomes.

Four systems for specifically and conditionally killing the pig hepatocytes will be developed and compared. This will include two mechanisms of expressing the suicide gene in the pig and two combinations of suicide gene/prodrug.

The suicide gene may be expressed ubiquitously or limited to the liver. Using the CMV promoter, the suicide gene would be expressed in all cell lines. The specificity would be achieved by using immunoliposomes with surface antibodies specific for hepatocytes. The advantage of this system would be that it could be applied to other cells in the future, such as endothelium and smooth muscle cells. One would only need to change the antibody in the immunoliposomes. Also, if immunoliposomes are injected into the fetal pig, most of them would end up in the target cells. Diffusion back to the sow would be minimal and therefore the possibility of toxicity to the sow would be minimized.

Alternately, the suicide gene could be limited to expression in the liver by putting the transgene under the control of the albumin promoter. This would limit toxicity to other cells cause spite nonspecific of the antibody in the immunoliposomes or from bystander effect, where the activated prodrug is passed from the target cell to adjacent cells. This would also allow for treatment of the patient for certain infections. For example, ganciclovir, the common prodrug for thymidine kinase, is also of the principal treatment for herpes infections. If the hybrid liver contained porcine cells with thymidine kinase, such as endothelial cells, those cells might be destroyed if the recipient were treated with ganciclovir for herpes infection.

The most widely used suicide gene/prodrug system is thymidine kinase and ganciclovir. Cells expressing this enzyme convert the ganciclovir to the triphosphate derivative. When incorporated into the DNA, the cells die. Generally, the activated triphosphate derivative passes to other cells only through tight junctions. Therefore, injury to endothelium and Kupffer cells would be minimized.

Injury to the liver of the gilt or sow could be prevented by breeding a homozygous transgenic boar with a non-transgenic sow or gilt. Only the fetal pigs would express the suicide gene.

Another widely used system is cytosine deaminase and 5-fluorocytosine. The prodrug is converted to 5-fluorouracil. When incorporated into DNA, the cell dies. This system would have advantages over the thymidine kinase system. Cell death is somewhat slower. Therefore any toxicity from the lysis of the native pig liver cells would be decreased. If used in the ubiquitous expression of enzyme, the patient could still be treated for herpes infections with ganciclovir. The downside would be that the activated drug could pass into adjacent cells through simple diffusion, thereby increasing the risk of toxicity through a bystander effect.

Overall Strategy

Even with the enhanced efficiency of transfecting oocytes with a retroviral vector, the production of transgenic pigs is a very time-consuming process, requiring approximately 18 months before transgenic pigs can be tested. If the four systems were tested sequentially, the plan would require approximately eight years. Therefore, we are taking advantage of the enhanced efficiency by producing four herds of transgenic pigs at the beginning of the project. As these herds are being developed, the systems will be tested and compared using *in vitro* tissue culture studies and transgenic mouse studies. During the course of the studies, one or more of the systems may prove to be infeasible. The development of that transgenic herd would then be discontinued. It is anticipated that two of the four systems will prove to be superior and warrant further assessment.

During the first year, the vectors will be produced. While waiting for those, the optimal conditions for transfection of porcine oocytes and implantation will be determined. Human hepatocytes will be infused

From 3/7/00 ATP g.m.p.

215

6/20/99

Sow # notches - left ear

✓ 203

✓ 187

✓ 176

✓ 152

P.lets in 187 and 152 have diarrhea. Have lost 5 from litter 152 and 3 from litter 187. Will probably lose some more, but seem appear to be gaining weight. Gave remaining p.gs O.S. and Tricell and 5 ml sterile saline IP.

P.lets for 176 and 203 are doing well. Lost one from each, # 176-9 and 203-8

The first two litters last week, 203 and 176 had no diarrhea. Those two litters were delivered as soon as the foster sow delivered.

The last two litters, # 187 and 152, had cons. derable diarrhea, though I have seen worse. At about 48 hours, we still have about 2/3 of the p.gs. I have seen the entire litter killed at 24 hours. For the last two litters, foster sows were brought in that had farrowed within 1 day. The designated sow had not delivered by Friday. The litters had to be delivered on Friday since Noel was leaving town on Saturday. The use of foster sows that had just delivered was only partially successful. The p.gs got the tail end of colostrum and probably not enough colostrum.

6/20/99

W. H. H. H. H.

475 (12/21/98)

J. H.

7/21/99

Sheep	1	100	65
Infected	0.32	0.37	0.45
Depleted	0.86	1.30	0.67
PBS	0.79	0.30	1.34
DNase	.01	.01	.012
Penicillin	.02	.02	.025
Total	2.00	2.00	2.50
Pigs	8	8	10
Infected/Total	1:3	1:3	2:3

7/22/99

The above 3 mixes contained an error.
 #100 had 2.5 ml, 1 + 65 2.0 ml.
 Furthermore, very few mononuclear cells were present in 100.

Decided to make new mixtures today following the same protocol.

#1 32/33 97% 5SS 1:20 3.2×10^7 / ml $10^7 = 0.31$ ml.
 #100 24/24 100% 5SS 1:20 2.4×10^7 / ml $10^7 = 0.41$ ml.
 #65 43/44 93% 5SS 1:20 4.3×10^7 / ml $10^7 = 0.23$ ml.

Sow #315 Received cells sheep #1 (0.31 ml ea)
 CR 6.4 cm. 6 p.s.s. injected.

Sow #316 CR length 6.5 cm
 Hepatocytes obtained from Steve Strain thru
 ILS for. Thawed just prior to injection.
 69% viable 26.3×10^6 total in 1.5 ml (1.75×10^6)
 Gave 8×10^6 to each of 2 p.s.s (0.45 ml). In these
 0.2 ml injected into liver, the rest into the peritoneum.

475 (12/21/98)

7/22/99

Sow 316 - Gave 0.15 ml into each of 2 p.s.s.
 injected into the liver (2.65×10^6 / p.s.s.) - stress
 Ira says the cell count refers
 to total cells, not viable cells.

Sow 105 CR length 5.8 cm, Sheep 100
 Injected 4 p.s.s. ≈ 0.4 ml, 1 p.s.s. ≈ 0.2 ml

Sow ~~105~~ 173 No live p.s.s. seen. Good size ≈ 6 cm.
 P.lets Recently deceased.

None injected. This sow was brought in recently
 for the hepatocyte study. Possibly the last 4 stress k.illed the p.s.s.
 Also it appeared that they gave the Varcomycin too fast - may have
 killed the p.s.s.

Specific gravity of Ficoll Hypaque.
 The ficoll of sheep marrow has worked
 well in the past, but now it doesn't work.
 Most of the nucleated cells sink below the
 ficoll hypaque layer to the red cells.
 Is the specific gravity less than the stated value
 of 1.077?

Large bottle (≈ 250 ml) Sigma Ficoll Hypaque, lot #39H2357
 Rec'd 5/13/99. Opened previously.
 Measured 10.0 ml of F-H and weighed it

1) 10.5242	2) 10.6185
- .0035 (boil)	- .0025
10.5207	10.6180
SG = 1.052	SG = 1.062 Ave. 1.057

Opened small bottle of Sigma Ficoll Hypaque lot 49H2303

1) 10.5613	2) 10.60642
- .00316	+ .00605
10.55795	10.61247
SG 1.056	SG 1.061 Ave. 1.058

7/23/99 42 E.B.

Injection of sow's hepatocytes

9/27/99

Sow	CR	Sheep	Volume	Pigs injected	
46	6.8cm	133	0.25	6.7	5
361	7.5cm	Hepatoocytes*	2-0.5	5	1
43	9.2cm	176	0.35	5	1
45	8.5cm	208	0.35	5	1
					2

* Inactivated (Naturally) human hepatocytes from Dr. Fox's lab. 100 million hepatocytes in 2.5 ml D. & 2 @ 0.5 ml (2×10^7 cells) and 3 @ 0.25 ml (1×10^7 cells) Ultrasonically of sows #105, 316, 315 doing fine, good growth, fetal movements

3 Days

8/30/99

Sows doing fine. No POC or discharge.

Sow #46 passed one fetal pig + placenta 9/1/99
Clear vaginal discharge. 11cm CR, 42 grams, clear amniotic fluid
Extremities echynotic, abdominal region echynotic.

No more fetuses passed from #46. 9/2/99
All other sows doing well.

As of yesterday, no more abortions 9/8/99
Assume #46 still has pigs. If 7 injections made into 5 pigs, 3 injected pig still left

?

#105 Sow

3/29/99

Delivered by C-section - W Johnson, C Sheara, T Yang. Delivered 7 full term live births, 2 stillbirths. Put on foster sow, who delivered 20 hours earlier. Gave each pig 1-2 ml cow colostr.

#316 sow (hepatomyel)

C-section - W Johnson, C Sheara, T Yang. Delivered 8 live births. Healthy nursing.

10/1/99

All piglets doing well other than 315-6, who was crushed, have not lost any more pigs.

10/3/99

Piglets from #316, now on foster sow 799 look thin, some yellow diarrhea spots seen on meat. Gave each pig 0.7 ml Penicillin IM.

10/4/99

Real thought piglets-316 had diarrhea. Gave 1 ml Pen to each pig (300k units!). Drew blood ~ 6 ml for serum.

[Drew blood from piglets on 105 (7) + 315 (2)]
All doing well & gain in weight.

Gave sheep #100 Cyclophosphamide - saline, 35 mg/kg. Weighed 6 kg. Recovered OK.

10/5/99

Gave sheep #1 Cyclophosphamide 53 kg. 35 mg/kg

Lost 4 pigs from 799 (316 -) 1 was crushed. Two died malnourished diarrhea. 1 euthanized - diarrhea

10/7/99

P.S.S 316 - 3, 4, 6 Fed 3x per day, v.a bottle
every 8 hours. 2-3 502 son replacement
formula. Appears to be gaining weight, stay.

10/11/79

P.S.S are drinking from dish.

Scott Thompson tested initial sera for
herpes - seen and fed.

Reported definitely positive

First # 2, 4 highest ~ 1:20000 a panel to him

Third # 6 ~ 1:30,000

Fourth # 5 "

Fifth # 1

Sixth # 7

Seventh # 3

Eighth # 8 - uninterpretable

Injected 4 p.s.s
2 high dose, 2 med. dose

3, 4, 6 still doing well.

gordyich polz Page 5, 10, 40
 PROJECT Albani ELISA human @ 1,5,25,000 Notebook No. _____
 Continued From Page _____

41

8/2 plates coated w/ dilutions by to .002g/well
 on Friday 7/30 - in fridge
 out of fridge 3:31 - sat started while mixing
 washed x2 3:56 - 4:03

Sub seen in 4:03 - 4:27 - some wells dried out?
 all human 1st, then control (neg),
 then experimental last

washed x3 4:57 5:03 - 5:10

HP made - 400 - 8ul - 20ul, 1,000 - 10ul - 10ul, 4,000 -
 52ul AP in 5:10 - 5:15 10ul - 25,

wash x3 ~~5:15 - 5:22~~ 5:55 - 6:03

HP in substrate 10ul in by 6:05

H₂SO₄ in at 6:10

trough was turning colors - didn't wash
 good enough

poly?

1

Continued on Page

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Date

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Date

OBJECT Alb. ELISA

113

Notebook No. _____

Continued From Page _____

3/4 plates out at 1:15

Seal mixed in Fish plate 1:25-1:40

washed x3 1:42-1:47

52ul Serum in by 1:49 - incubated til 2:20

52ul Serum in by 2:10 in 2 other plates
had to add db on top at _____ incubated til 2:43
(row 2) 2:30

washed plate 1 x3 2:45-50

2 Hr in 2:50-2:55

washed other 2 x3 3:00-3:05

52 Hr in by 3:10

washed plate 1 3:40- x3

100ul substrate in by 3:42

100ul " " 3:51 in other 2

H₂SO₄ 1- at 3:5

2

Continued on Page _____

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Date _____

PROJECT AB - ELISA - diff. wash and block

8/6

* 3.1% sheep = 25ml
 .8ml

plate out 11:10

(2 1/2) -
 washed x3 - bottle - 11:21 - 11:24

blocking * 3.1% sheep (94-10/29) - 100µl in # by 11:

Left columns 7-9 w/our liquid (covered) until
 added last wash w/ pipettas at 11:45 (105µl)

washed 13 columns 10-12 11:57 - 11:59 (105µl)
~~bottle last wash~~

mixed sera in Nunc plate (same as is coated)

Same dilutions as 8/4 in tubes, 100µl ds/200µl
 (only made sheep buffer)

52µl sera added by 12:04 3A got ds + bsa

Incubated 12:10 - 1:25 (covered)
 (CO₂)

washed x3 (125) 1:33 - 1:37
~~bottle last wash~~

52µl HP in by 1:43

Incubated 1:45 - 2:48 (uncovered)
 (CO₂)

~~100µl substrate in after~~
 wash x3 (125) 2:51 - 3:00
~~bottle last wash~~

100µl substrate in by 3:04

H₂SO₄ in at 3:12

3

Continued on Page _____

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Dr. _____

Immulon - carb/bicarb 1 plate PBS 1 plate - Cab

OBJECT Alb - poly/poly sandwich immunoassay

Notebook No. _____

Continued From Page _____

3/10

trying different coating buffers on different plates
and different blocking buffers, diff ≤ 7 s Cab
rashed x3 prior to coating

10:00 coated 4 ~~plates~~ blocking: Stabilizer, pH 7.5, PBS
(see Template)

Sera dilutions: See Template

Immulon plate runs over more than 1 hour while using
bottle wash method

10:40-10:45 wash x3

Sera in at 11:00

wash x3 11:35-11:42 (3 $\frac{1}{2}$ wash let sit complete minute)

HP abs 10ul/10ul, 2ul/10ul PBS Tween, 2ul/3ul PBS

52ul H₂O in at 11:50 (1,000/4,000/10,000)

wash x3 12:23-12:28

100ul substrate in 12:30

50ul H₂O in 12:40

plate coating: 7.15 μl in 5ul then 1ul to 9ul next 2 ≤ 7 s

3 $\frac{1}{2}$ 3 10H got 20 sera \rightarrow
Box + 1 $\frac{1}{2}$ sera \rightarrow

8,9,125 got 40 sera \rightarrow
added 5ul 2000
Continued on Page 40200

Read and Understood By _____

Carb - Nunc

PBS - Immunolon

Notebook No. _____

PROJECT Ab - poly / mono / avidin - HPO

Continued From Page _____

9/10

no blocking buffer, PBS/Tween for ab

2:00 - 2:10 wash x 3 - pipette washes

52ul sera in 2:20

wash x 3 3:00 - 3:06

(6ul in 10ml) - should be 5, but

52ul mono abs ($2,000/8,000$) at 3:10mixed biotinylated anti mouse ab - $(1:1,000)$ - 10ul/10ul

52ul biotinylated added at 3:30

wash x 3 at 3:50 - 3:55

52ul biotinylated added at 3:58 ($1:1,000$)

wash x 3 4:30 - 4:37

52ul avidin in at 4:40 ($1,000 + 2,000$)

wash x 3 5:15 - 5:20

100ul substrate in 5:23

52ul H_2SO_4 in 5:33Accident 12C, E, H on Maxisorp

Accident 12C, E, H on Maxisorp

Continued on Page _____

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Date _____

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7/15

coating plates w/ serum

5mls of each { } for Pig ⁴⁰⁰ 50, 100, 200 HS ~~2,000, 8,000, 24,000~~
^{125ml} ¹ ¹ ¹
~~125ml~~ ~~50ml~~ ~~25ml~~ ~~25ml~~

HS 2,000 - 8ml / 4ml 4.5ml

8,000 - 2ml (2,000) / 4ml

24,000 - 2ml (8,000) / 4ml

mixed in PBS + Carb

50ul HS, then immediately 50ul Pig
 100ul of straight serum

straight serum was doubled from 2,000 to 4,000 etc. +
 after mixing HS + PS in wells 8,000 to 16,000
 nothing done w/ 24,000
 all pig serum doubled

7/17

wash carb (Immunon ~~Maxisorp~~ + Maxisorp) plates x3 w/ 100ul

monoclonal anti-HSA mixed 4ul / 20ml wash buffer (1/5,000)

100ul monoclonal added 9:50

wash x2 10:20 - 25

100ul biotinylated abs in at 10:30 - 2ul / 20ml

mixed avidin - 21ml / 14ul (1/1,500) (1/1,000)

wash x2 10:55 - 10:59

avidin should be
 4x higher probably

100ul avidin in at 11:01

✓ [] *

wash x2 (maxisorp 3rd one w/ bottle) 11:26 - 11:33

100ul substrate in 11:35

52ul H₂SO₄ in 11:41

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Date _____


Signed _____

9/22 wash x3 ~~100~~ 120 μ l 8:25 - 8:33

monoclonal anti-HSA 2 μ l / 20ml wash buff
(2.2) (1/10,000) (9,000)

wash x3 120 μ l
100 μ l monoclonal 8:35

wash x3 120 μ l 9:10 - 9:15

 E-H A-D

100 μ l biotinylated abs in 9:17 - $\frac{1}{3,000} + \frac{1}{6,000}$
(3.3 μ l / 10 μ l) (1.7 μ l)

wash x3 120 μ l 9:55 - 10:00 CD, EH AB, EF

100 μ l avidin in 10:04 - $\frac{1}{2,000} + \frac{1}{4,000}$
(5 μ l / 10 μ l) (2.5 μ l / 10 μ l)

wash x3 120 μ l 10:45 - 10:50

100 μ l substrate in 10:53

52 μ l H₂SO₄ in 11:00

9/24 coating plates of experiments - see Template

coated 4 plates from 5-6:30 (1 1/2 hrs)

2 munc, 2 Immunon

50 μ l H₂O / 50 μ l PS or 100 μ l straight sea

used only PBS

Continued on Page

Read and Understood By

7

Signed

Date

Signed

Date

sh x3 120 μ l 9:08 - 9:1810 μ l monoclonals in at 9:19 - 2 μ l/20ml wash buffer
(1/10,000)wash x3 120 μ l 9:54 - 9:5810 μ l biotinylated in at 10:00 - 2.5 μ l/20ml wash buffer
(1:8,000)wash x3 120 μ l 10:35 - 10:3910 μ l avidin/HPO in at 10:40 - 4 μ l/20ml wash buffer
(1:5,000)wash x3 120 μ l 11:15 - 11:2010 μ l substrate in at 11:2152 μ l H₂SO₄ in at 11:343 2 plates out of fridge - air dried - no blower
't.i' 10/15 (40 hrs)

1.5 wash x3 - bottle wash 9:21 - 9:33 Nunc

10 μ l monoclonal in 9:35 - 2 μ l/20ml wash buffer
(Nunc) (1/10,000)

wash x3 - bottle - 9:32 - 9:43 - Imm

5 μ l monoclonal in Imm 9:445 μ l biotinylated in Imm 9:46 - 2.5 μ l/20mlwash x3 120 μ l 10:07 - 10:11 - Nunc10 μ l biotinylated in Nunc 10:12 (1:8,000)

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8

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Date _____

PROJECT _____

Continued From Page _____

wash x3 120 μ l 10:40 - 10:47 both101 μ l avidin/HRP in at 10:49 - 4 μ l/20ml (1:5,000)wash x3 120 μ l 11:19 - 11:25100 μ l substrate in 11:27 Imm 11:28 Nunc52 μ l H_2SO_4 in at 11:3810/15 Made plates - boiled sera PS + #2 - glob - need
to find different way
#2, 4, 6

10/18 wash x3 - both 9:20 - 9:30 - just Imm plate

101 μ l monoclonal in 9:33 - 2 μ l/20mlwash x3 120 μ l 10:06 - 10:12101 μ l biotin in 10:13 - 2.5 μ l/20mlwash x3 120 μ l 10:44 - 10:47101 μ l avidin/HRP in 10:48 - 3.9 μ l/20mlwash x3 120 μ l 11:18 - 11:22

Substrate in 11:23

 H_2SO_4 in 11:31

10/20/92 Try plates made 10/15 (dried) w/ polyclonal at (1:5,000)

washed

100 μ l poly in at 9:35 - 10:30

Continued on Page

Read and Understood By

9

Signed _____

Date _____

Signed _____

Date _____

OBJECT _____

Continued From Page _____

10/27 heated pig sera to 65°C for 30 minutes

10/29 SAA - 316 pigs (see Template)

Dis buffer 3ml / 20ml PBS
 (10x)

rehydrated plates (washed) ~~5:30~~ 5:30 - 5:55 x3

50µl sera, then 50µl 2° ab in by 6:00

★ Next time add 2° ab ^{1st} so no chance of getting sera on tip and moving to next column

2° ab 11µl ab / 27ml db (1:700)

HS: 10µl / 5ml db (1:500)

SAA Standard: 20µl stuff / 180µl db

Wash x3 6:58 - 7:05

100µl substrate in by 7:06

out at 8:05

Continued on Page _____

Read and Understood By _____

PROJECT _____

(11/8)

11/11 SAA - 361 1-7, 316-3, 6 (11/28)

Mixed wash + dilution buffers = dd H₂O

See 11/11 Template -

Standard 10 = 20 μ l / 180 μ l dil buffer

HS 500 = 100 μ l db already in well then
HS 250 = 10 μ l / 4 ml dil buffer

2° ab 700 = 10 μ l / 7 ml db

PS 2 = 100 μ l db already in well then 100 μ l

11/19 SAA - 316 3, 4, 6: St, HS, PS, 3(11-8), 4(10-7), 4(11-8), 6(11-8)

1/12/00 2° ab 500 = 20 μ l / 10 ml db

St, HS, ^{HS}PS, PS, 361 1-7, 316-3 - plate 1

all wells in Row A
control db

St, 316-4+6, 361-1, 366-4 - plate 2

✓ 12/17 + 19

HS = 311-1

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Signed _____

Date _____

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Date _____

Beschoner, W.E. Proprietary Information 03/07/00
Human/Pig Hybrid Livers for Transplantation

**Proliferation of Human Hepatocytes in Pig Spleens
And Detection of Human Liver Proteins**

<i>Piglet</i>	<i>Hepatocytes in the Spleen</i>	<i>Est. Expansion of Injected Hepatocytes*</i>	<i>Human Serum amyloid A#</i>	<i>Human Serum AAT#</i>
75-1	3.6x10 ⁷	18X	0.12%	0.9%
75-2	1.6x10 ⁸	90X	0.23%	22%
75-3	5.8x10 ⁷	29X	0.10%	5.5%
75-4	7.6x10 ⁷	38X	ND	ND
75-5	1.6x10 ⁸	80X	ND	ND
316-1	ND	--	0.90%	ND
316-2	ND	--	0.73%	ND
316-3	ND	--	0.24%	ND
361-1	ND	--	0.45%	ND
361-2	ND	--	0.52%	ND

*Number of splenic hepatocytes/Number of Injected Hepatocytes

Concentrations compared with concentration in human control sera.

The second and third sows produced six and eight live births. Serum analysis for SAA and alpha-1 antitrypsin were consistent with chimerism in two and three pigs respectively. These pigs have been followed for up to three months. At three months one of 2 pigs (316-2) shows persistent human proteins (0.6% SAA). That pig currently weighs 20 kg and is clinically normal and healthy. At 60 days of age, three of 4 evaluated pigs had human SAA in the serum.

The preliminary findings, therefore, support the proliferation and function of human hepatocytes in the fetal and newborn pigs. The injected hepatocytes expanded at least 18 to 90 fold and produced human proteins. The detection of human liver proteins in the serum confirms the presence of viable hepatocytes in the pigs. Furthermore, the localization to the spleen and the failure to identify hepatocytes in the liver is consistent with the hypothesis that the infused hepatocytes are competing with native hepatocytes.

Transfection of Porcine Renal Epithelial Cells.

By introducing a retroviral vector into oocytes rather than targeting embryos, Chan and Bremel were able to achieve a very high efficiency of transfection and avoid the problems of mosaicism⁸. The transfections were performed with bovine oocytes.

To determine the feasibility of this technology with porcine cells, a cell line of porcine renal tubular epithelial cells (PK-15) was utilized. The vector was a replication defective vector based on the Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus. The vector included a neomycin phosphotransferase and a reporter gene (GFP, green fluorescent protein). The PK-15 cells were transfected with a high titer of the vector and the resistant cells selected. The cells were cultured for three weeks. They demonstrated diffuse expression of the GFP.

Prevention of Xenograft Rejection with Surrogate Tolerogenesis.

As an alternative to inducing immune unresponsiveness *within the recipient*, we propose instead to induce immune tolerance to the donor *within the xenograft donor*, and adoptively transfer the tolerance back to the recipient. We have termed this process "surrogate tolerogenesis"³⁸. The induction of tolerance within the donor animal provides considerably greater flexibility and opportunity. For example, with current approaches developmental tolerance could only be applied to fetal or newborn patients. With surrogate tolerogenesis, however, developmental tolerance can be established within fetal donor animals

From 3/7/00
ATP grant

EXHIBIT 19

SAA History of 361 and 316 litters

Pig #	10/4/99	10/8/99	10/18/99	10/27/99	11/8/99	12/17/99	12/19/99	1/12/00
316-1	100							
316-2	500-700	200						
316-3	70		70	neg	neg		neg	neg
316-4	420		20	300-600	*		neg	neg
316-5	130							
316-6	250		20	20	neg		neg	neg
316-7	100							
316-8	neg							
361-1					60	50-70		neg
361-2					130	neg		*
361-3					70	neg		neg
361-4					120	neg		neg
361-5					160-200	*		*
361-6					*	neg		neg
361-7					170	20-30		25

All values are in ng/ml

* Denotes a possible positive that is not statistically significant

316 delivered 9/29/99

361 delivered 11/4/99

PROJECT _____

4/4/00 Permann B2C1 (12 tubes) PCR again due
to not enabling heated lid last x. 3ul of
DNA used in each rxn. No mineral oil.
440(217/00) as SC. Red APC as pg control.
A lot of protein in samples, if doesn't work,
attempt proteinase K digestion.
Using 3ul due to use of 5ul in first
attempt (3/14/00 results).

* All red APC rxns received 4.5ul DNA instead of
3ul. 55°-59° S. 10,000 received 4.5ul DNA
instead of 3ul.

Gel ran 2:20-3:30

Seq's 57°-60° (+), All others negative
due to too much DNA? Will dilute
DNA samples. 57° band is brightest -
(in my research tubes)

Continued on Page _____

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1

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Date _____

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4/5/00

Different Different dilutions of DNA w/ revised
 SHEEPSCT: Anneal 57° (61°), Extended 72° (70°), cycles 50 (45)
 440 (27) or 2 dif. dilutions 27 μ g/l + 18 μ g/l
 (3 μ l) (2 μ l)

Dilutions red APC (450 μ g/l)

20 μ l APC + 20 μ l \rightarrow 200 μ g/l

then 20 μ l + 20 μ l $\times 3 \rightarrow$ 25 μ g/l

20 μ l 510,000 + 73 μ l \rightarrow 200 μ g/l
 (930 μ g/l) 73 μ l

then 20 μ l + 20 μ l $\times 3 \rightarrow$ 25 μ g/l

tubes: APC, 10,000, 100,000, SC + 1 mm, + 2 SC

$\times 4$ []'s of 200, 100, 50, 25 μ g/l
 except SC at 10
 27 + 18 μ g/l 27 μ g/l
 (3 μ l) (2 μ l)

Only SC volume 3 μ l \oplus

Try different MgE7s from 55-60 $^{\circ}$ in Rebo

2 μ l to 125 μ l red APC - incubated overnight

Continued on Page

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PROJECT _____

Continued From Page _____

4/6/00 ~ ~

Talked to Cara, (Genet Tech manager, -
 did you need blank w/ hydration ^(HB) buffer? No

Measured DNA after blanking w/ HB in HB
 and in dH_2O - By difference

A_{260}/A_{280} ratio now 1.7 instead of 1.1-1.5 -
 also reason is higher A_{260} not absorbance

giving higher E DNA keep blank as HB

Digested ~~AluI~~ ⁵¹⁹⁰⁰⁰ (200 μ g/ml) with $\text{I} \mu\text{I}$ Not I
 for ~~2 hrs~~ ^{2 hrs} at 37° (gc/ggcgc)
~~1 1/2 hrs~~

Ran 24 tubes in Robocycler from $64^\circ - 60^\circ$ -
 noticed after mixing runs that the gradient
 block is the reverse of what I thought.
 Best was not 57° but 64°

My E 's from 1.2 - 1.8 included, + Tween at .5%
 All negative - 4/7/00

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Date _____

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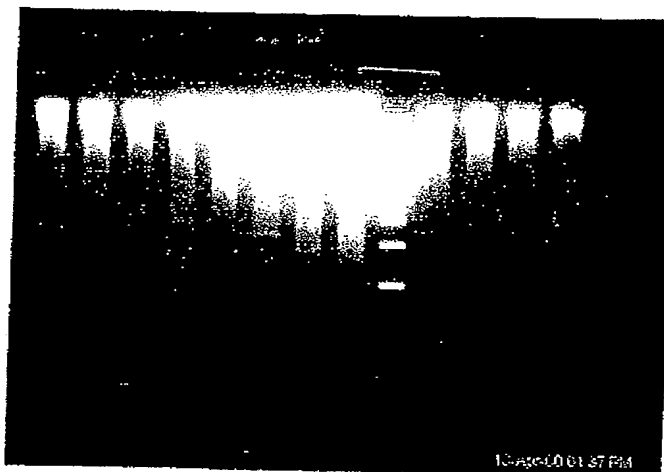
Date _____

4/7/00 Averted after
Cycle across hall

1° run 4m at 96°
45" at 95°
B2C1 25" at 63°
30" at 72°
42 x 5

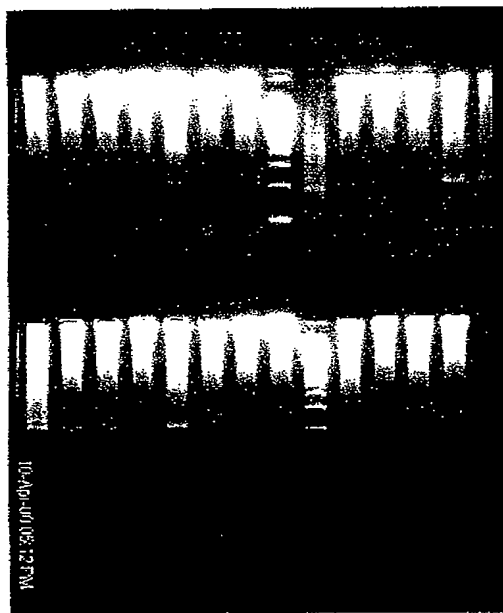
W250L : 40" at 93°
25" at 6

B2C1 R
B2C2 F



1°

4/10/00 Nested up products from 4/7/00 using B2C F2
(B2C2 F) and B2C R1 (B2C1 R) - 2nd of products at 62°



used

① (lane 14 - with glycerol brighter)
re 15 - 2.5 µl of 1:1,000 (blood: blood)
at 1.2 mM $MgCl_2$
2 - 3 µl of 1:1,000 (blood: blood) at
1.6 mM $MgCl_2$ w/ glycerol (10%)
lane 6 - error in components?
band? also - 1st run didn't work?
↑
lane 14 + 10

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4/25/00 All samples from 4/24 were \oplus using
 Hoopes + Plate primers, So looking for
 contamination or program differences. Today,
 tested programmed cycles according to
 Hoopes + Plate protocol at 55° for 20 sec (anneal)
 Also, using de puregen and MT tubes,
 sterile vs ddH₂O, and different hydration buff
 in mM

tube #	1	2	3	4	5
ddH ₂ O MT	SC	P1,000	MM A	MM B	SI
ddH ₂ O puregen	SC	"	"	"	"
sterile H ₂ O MT	SC	"	"	"	"

Only sterile H₂O SC tested \ominus , however,
 no master mixes tested \oplus : Interaction between
 carbon filtered H₂O and ship DNA?

Attempted 441 run for pig microchip again
 SC, P1,000, P10,000, x, x, x, x, x, x, x, SI, MM
 (193) (208)

4/26/00 Same samples as yesterday used in same
 PCR program, same cycles, same ETs. Run
 on new gel. Yesterday old gel used. Samples
 in same order.

Continued on Page

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5

4/24/00 Diluted dNTPs x 5 tubes

288 μ l TE + 6.25 μ l each dNTP

⊕ (need to ✓ TE pH)

Diluted MYF primers with TE: MYF F, MYF R1,
MYF R2

SC from 4/19/00 was ⊕ for pig -
either increase temp or DNA contamination
try Robocycler tomorrow?

4/27/00 Using Robo 56°-59° using Hanger + Plaster primers
w/ P1,000 and P10,000 - (12 samples)

also trying new Shae primers: SC + APC
used at 11 temps. 53°-63° + using MYF F / ~~MYF R1~~
and MYF F / MYF R2 (22 samples for each
program 71) 44 total samples)

Results: Hanger / Plaster blurred ⊕s w/ P1,000 -
when loading PCR tubes, it appeared as if
too much mm was left over. Do again tomorrow

pH of TE made by NT is 7.8 - (7.6)

MYF F / MYF R1 - very good 53°-55° bright

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54° in APC has sharp band and 59° has smaller frag. - these could be contamination - checking results of R2 set before any conclusion

4/28/00 Running gel on R2 set - Not as good

Program 71: 95° 2min
95° 25'
53-64° 25' > 40 cycles
72° 35'
72° 2min
6° 99min

55° + 61° bright ⊕s

53° + 60°, 62° light ⊕s

absolutely no false ⊕s in APC row

Running Rebo w/ Hynes + Place again and MIF F/MIF R (same as yesterday)

53° - 56° w/ 5,000, APC, 5,10,000 + 1 SC

Program 72: 4 extra cycles

Made ELISA wash buffer (1 liter) - PBS + .05% Tween

" dilution buffer (100ml) - Wash + 1% BSA, S
(Exp 3/00)

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5/1/00 Talked to Ruback about 4/28/00 PCR -
 Hedges + Platt - no false \oplus s of APC at 56-59.
 M/F F/R exhibit different bands at different lengths
 depending on CT. 1/1,000 same as neat solution,
 but 1/10,000 smaller + larger bands at different
 locations - Ron doesn't have an explanation.

Began extraction of S155 final spleen (.0135g)

S330 OES embelid spleen (.0125g)

S440 " (.0125g)

used protease K incubation on all starting
 at 3:15 pm overnight.

5/2/00 continue extractions - unknown if any
 DNA recovered from S155

Set up IgG ELISA

New plate Template made - including dilution factor
[S=210 dil] - formatted to blanks up 8 standards [Is

	1	2	3	4	5	6	7	8	9	10	11	12
Plate A	1/12	1/19	161(1/29)	191(6/12)	441(2/4)		441(2/7)		441(3/2)		191(6/17)	
Plate B			263(2/27/9)		3(6/19)		3(6/22)		147(6/19)		147(6/24)	
	263	263		34(6/11)								

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Mixed sera in Nunc plate: 28.5 μ l db, then 15 μ l sera
 Standard 263 (1/15) in plate A 1st (1:20) in H
 in plate B last

(to see if difference after
 15-25
 sitting in plate for 30 minutes)

200 μ l added to 100 μ l db from 6-A

Rehydrated plate during this procedure from 1:00-1:30
 (7/15)
 rinsed x1

50 μ l sera from Nunc plate transferred to 7/15
 (25,000) plate at 1:35

Agitated x1 for 10 sec at 2:25

washed x3 (2:45) - 2:55

50 μ l HP in at (2:55): 16 μ l db / 5 μ l HP (1:3200)

Agitated x1 for 10 sec at 3:20

washed x3 (3:50) - 4:00

Substrate in at (4:00) 24 μ l citrate/phos, 24 μ l H_2O_2 , 6 tabs

50 μ l H_2SO_4 in at (4:20)

Need to begin dilution at 1:10 (only need 6 dilutions)
 (1:5-?)

Continued on Page

* Standard at (1:1.25) or 1:2.5

Signed

Date

Signed

Date

5/3/00

Meeting 9-10:45 - hire someone? do IgG ELISA w/
transplant controls (unsensitized)

PCR - Hanger/Pho program 72 on cycle across heel

95° 2min

95° 25'

56° 25'

72° 35'

72° 2min

5° 99 hrs.

> 45 cycles

Talked to Ron - could try to do 2 amplifications
w/ same primers for more sensitivity - he
warned about artifacts however

PCR lanes 441 2/4, 2/7, 2/14, 2/21, 2/28, 3/6, 1,000, 10,000, 3/14, 3/28, 4/3

SC, 440 2/1, 2/7, 5plus, 1,000, 10,000, 320, 155, min

> maybe try 30 + 30 or 35 + 25 or 45 + 30 etc

5/4/00

Talked to Ron about false positives in
Hanger/Pho - contamination from blood, labcoats, pipettes,
gel, etc.

To need to draw blood at Dan Melnik's -
draw 35 cc's from 101

draw 2 uls from 329 piglet - same as previous

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2 or 3

Name

Signature

Date

PROJECT _____

Began extraction of SC (101), P10,000 (322),
and from experimentals: 320 - 2/3, 2/7, 2/10
155 - 2/7

2.5 - 5.0 ml of exp. blood
(frozen)

used 11mls in SCs to be named SC10 + SC

used all new Puregene solutions

P10,000 mixed 1:9 \$325 blood/PBS then 9.5ml

SC(101) w/ 10 μ l dilution

P10,000 named [P10]

5/5/00 continued extraction - didn't recover much
DNA from 155 2/7/00, but definitely some.
Added ~ 600 μ l to SAS + P10,000, less to
experimentals (P10)

- Printed online inventory, organized in binder

ELISA - need to try different options to extrapolate
from standard ~ dilute standard 1:1.25 w/ 8 dil.
Table for ELISA

Continued on Page _____

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5/8/00 [TE] To 450 μ l e-pure H₂O - (Sigma E-4884)
 EDTA - disodium salt, dihydrate - 18.6 g EDTA
 (372.2 mmol)

brought up to final volume
 Adjusted pH w/ HCl + NaOH to 7.60 g 500ml
 6055g Tris (127.1 mmol)
 (Sigma T-1503)
 autoclave

Using S11 - ~~precipitation~~ aliquoted into 3 tubes
~~S4-S6~~ 53-56 - H₂O/Plat Robo - see 4/28 template

53-56 H₂O/Plat Robo - see 4/28 "

Performing same PCR as 4/27/00 with program 72

~~Diluted~~ Added 120.1 μ l TE to MYE FN primers
 received 5/5/00. Diluted 1:20 380 μ l / 20 μ l

5/9/00 Ran gel from 5/8/00 PCR +
 negative controls on H/P positive at 54 + 56°
 negative controls have bands in MYE at 56 + 55°
 (lanes 2 + 5)

Did nested w/ both neat and 1:40 w/ H/P

See 5/9/00 template 1:20, 1:50, 1:100 MYE

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Place _____

- Made new solution TBE for gel chamber — w.c.
- make new TBE concentrate
- Gel loading dye aliquots from run
- different pipette for loading products

other possibilities: contaminated primers (from TE)

" RNase
pipettes

" DNA now
PCR tubes

Sometimes some DNA inaccessible?

5/10/00 TBE: To ¹⁵⁰ ~~100~~ ml e-pure H₂O - 37.22g (Sigma F-4
5M EDTA ¹ bring to pH 8.0 w/ NaOH pellets
bring to 500ml final volume

~~TBE~~
Run PCR on cyclo across lab - program 72
with H/P at 55° (all at 55°), M₁E F/R₁, and
M₁E F/R₁, at ran gel - only
M₁E F/R₁ had no false ⊕s

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5/11/00 JBE Tris (T-6791) 54g
 Boric Acid 27.5g
 to 900 ml expure H_2O
 then 20 ml ^{5M} EDTA (pH 8.0),
 then final volume 1000 ml

Cool from nested run performed after 1st yesterday run
 thinking that maybe annealing may be different in
 reboiler compared to cycle across hole due
 to temp change being immediate.

So, maybe H/P needs to be at 57° -
 will try tomorrow unless nested appears best

H/P 1st 7 lanes, nested MVE 8-14 then
 run 1st products from lanes 5-7 (SC, 10,000, SC)

from yesterday

lane 12, 13, 14 (10,000, APC, 100,000)

lanes 20, 23 (51,000, APC)

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5/12/00 Using program PIGH00 (anneal at 57° 20 sec)
on east counter/lab bench

~~re-running 5/3 H/P add SC after run in 2nd run~~
Same Template as 5/10/00

red APC/SCII/1,000/SCII/10,000/SCII/mm
(blue)

Using program 72 (55° anneal 25 sec) on
west lab bench (was run on east lab bench
yesterday 5/10/00 when successful) using MYFF/R

5,000/APC/50,000/APC/500,000/APC/mm

Results: MYF F/R again sensitive to 1:100,000
with no false ⊕s. H/P sensitive to 1:1,000
w/ no false ⊕s

5/13/00 running H/P on Robo at 56° and
with pigh00 (east bench) again - same primers and
only 1:10,000 w/ 2 neg controls.

PIGH00 (H) Robo (R)

HSCII/H Pigh00/HSCII/RSCII/R Pigh00/RSCII/mm

Results: Nothing

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Trig 5/3/00 over w/ 2 runs in Robo at 56° - 56°
in Gradient mod
bad? →

441 (2/4) / 2/7 / 2/14 / 2/21 / 4/3 / ^{black} P1,000 / ^{blue} P10,000 / SC11 / 320 (2/3) / 2/7 / 2/10

440 (2/1) / 2/7 / splen / 320 splen / 155 splen / P1,000 / P10,000 / SC11 / mm

* added ~~dist~~ c-pu = H₂O to SC11, 440.5, 320.5 accidentally
aliquoted SC11 into 2 other tubes

diluted PSC F2/R2 : 380 μ l TE / 20 μ l stock

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5/24

Serum protein content noted - need to add
10% - 20% more protein precip. solution and
spin for 150-200g. longer

Results PCR: No false +s

No samples +

All + controls +

Flow Cytometry all -

5/25/00 Extracted DNA from 300ul whole blood
no samples clotted; observed DNA in all tubes

1115 1-10, PC drawn today

2125 1, 2, 4-7, 9, 10

1191 2-5, 7, 8

1163 1, 3, 6-9

2126 1, 4, 5

2127 3, 5, 7

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Date

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Date

5/29 programs 57, 59, 57, 59, 61, 63

program 72 1° run

57° on west lab bench

59° on east lab bench

61° upstairs on 11th floor

wrong size block

44 x 5

61° on west bench

63° east " (63A)

1° run all 16 lanes (10 APC, PC) (5 100,000 - 50:2)
(+ master mix)

57° - false ⊕ also at 61° and 63°
59° -

Try 53°?

5/30 Tyler mixed 1° run, then,

I mixed nested w/ same primers and

ran on 57 and 59 - used pipettes from

beat 50:2 PCR tube

in program 59

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other tissue culture room

Signed

Date

Signed

Date

5/30 also run nested on Robo at 57° (program 57
 with no gradient) in 10 run from 5/29
 2125-7 1115-7
 5/30 104+101 APC PC forward primer
 2 transplants 50:2 w/ nested primer 1 cycle in 9th

5/31 Run gel on products from yesterday
 57 55 57 Robo
 ladder 1 2 3 4 5 6 7 8 9 10 11 12 13 14 ladder
 APC PC 100,000 50:2 M APC PC 100,000 50:2 APC PC 50:2
 (+)

all rest ⊖, run 1° run and
 all were ⊖ - even 100,000
 could be contamination - 5/29 stuff
 Need to do 5/30 over - make sure
 10 makes

Cytotoxicity assay failed

1 transplant (2126-5) SA 103

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Signed _____

Date _____

6/5/00 Tyler extracted 2 samples w/ 300 μ l to determine if enough DNA \rightarrow Yes (on samples from cord blood taken 6/1/00) T.
 continues to extract other samples.

I am running PCR on controls ($\oplus + \ominus$) to attempt nested PCR = APC PC

in (perkin-elmer tubes), on 100,000 50.2 M
 program 72 nest counter

~~* 1 $^{\circ}$ run again did not work~~

6/6/00 Summary PCR:

5/29 nested run worked, ? about 1 $^{\circ}$

5/30 1 $^{\circ}$ + 2 $^{\circ}$ did not work w/ 100,000 (1 pass \oplus)

6/5 1 $^{\circ}$ + 2 $^{\circ}$ "

Using perkin-elmer again today for nested runs
 ran 1 $^{\circ}$ yesterday - products saw in cycle
 overnight.

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1st results as should be - but very fast
 unsure if problem is ^{uv} bulbs in ~~uv~~ or
 nucleotides (Gibco - I think) ~~has~~ ^{had} been using Pongia
 from 4/26 but mixed others above I think
 led our uv and 13 and all look the
 same

Extracted DNA from	385 5, 10	using 3ml
1184-2	2141-1, 2, 11	card blood
484-1	1187-6, 7	- samples
395-1, 5		that T
		couldn't get
		DNA from

395-2 + 1187-10 - No DNA

Nested did not work up same primers
 nested primer on Robo gave false +s

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6/7/00 PCR on Samples extracted 6/5 & 6/6

mixed new solution of dNTPs Promega and
diluted both primers from stock

Picked up new dNTPs from GS

Also decided to change 1st run to eliminate
false ⊕s in 2nd run

↑ anneal to 58°C ↓ time to 20 sec.
(program 58)

will do nested at 57° w/ program 57

	APC	APC
1:1000	100:1000	100:3

False ⊕ in Tube A of both nested runs
but not tube B no spruing in one tube?

contamination?

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6/8/00 Running PCR on (program 72) samples and controls up mixed
 0.7 in program 59.20 PC APC
 1,000 100,000 75.3

Gel w/ samples - ⊕ controls did not always work

Extracted DNA from spleens (+K)

1163 - 3, 9

1115 - 5

2826 - 5

2125 - 7

6/9/00 PCR nested in controls and experimental samples - ⊕ controls still only ⊕ at 1:1,000

6/12/00 meeting - made chemicals up - stuck in auto clau, Troubleshooted PCR (nothing) (6791) Tr's

6/13/00 solutions had boiled over in autoclave - will make again

Tr's 2141-4 into 105

Went over ELISA protocol & suggested dilutions for Streptavidin ELISA

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6/14/00 Making Solutions Tyler 5M EDTA + TBE

TE: in 500ml grad cylinder - 450ml capure H_2O

T-6791 (Tris base) .6055g
E-4884 (EDTA) .186g

brought to pH 7.75 w/ HCl

Volume up to 500 ml and autoclaved -
overnight - lost H_2O due to evap.
~~removed immediately~~ - refrigerated

also made TBE + EDTA

6/15/00 replaced lost H_2O in TBE + EDTA

made TE as 6/14 (pH 7.75)

brought to 500ml and autoclaved -

removed immediately - refrigerated aft
in cool H_2O bath for 1-2 hr.

Tyler is performing anti-pig abs ELISA

trial w/ Streptococcus polymer - S263 11/29/99,

P 316-6 11/8/99. 1:100 Sin P then diluted 1:10

in dil. buff Diff [s] of db (.5% + 2%),

Strep (1-100,000), brain abs (1:10,000)

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Date

Made drafts of new IE 575 +12.5 each
2 aliquots

reconstituted new cartridge primers
and 2 aliquots 380 / 20

Tyler's ELISA - barely color change -
need to ↑ add biotin abs + streptavidin

4/19/00 Different C7s for my Ch₂, KCl with
new primers + new IE

9 diff. runs at 56° annealing (program 57 edit)
→ see PCR worksheet

Ficolling 17ml total of 385-1 blood

4ml Histopaque into 4 15ml tubes: PBS added

→ to 40ml and separated into the 4 tubes
lost some cells due to loose pellet w/ lots of RBCs

PCR tubes: 2, 6, 7, 9 lost H₂O
+ 4

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Date _____

6/20/00 higher primer @ Tm, lower salt [?]

Spleen PCR for sheep microchimerism
with 2x MgCl₂ (results from 6/19 establish this)

115-5, 2126-5, 2125-7, 1163-9, 1163-3, 385-1, PC, ⁵1,000, ³10, ³100
blood

Tyler attempts ELISA again w/ 1% Baking Soda,
1:1,000 / 1:3000 biotinylated, 500 / 1,000 / 5,000 / 10,000 Streptavidin

6/22/00 attempted diff. C₂ Buff + MgCl₂ w/
Promega solutions - didn't work well

Realized that maybe had used C₂
Solutions previously for mIF stuff (or
a combination of both) Try tomorrow

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Signed _____

Date _____

Beschoner, W.E. Proprietary Information 03/07/00
Human/Pig Hybrid Livers for Transplantation

the normal fetal pig, human hepatocytes would not be expected to engraft in the liver to a significant degree.

Fetal pig hepatocytes will need to be selectively destroyed, providing space for engraftment by the human hepatocytes. The concept of space for engraftment is well documented. For example, bone marrow transplants will not engraft unless the native bone marrow is first destroyed. This is seen even with autologous transplants. Therefore, it is not simply a matter of rejection of the new marrow. Space can be provided for bone marrow engraftment with total body irradiation or cytotoxic drugs like Busulfan. These measures however would not work to selectively destroy fetal pig hepatocytes. The hepatocytes are not as radiosensitive as other cells in the fetus, including bone marrow and endothelium. The radiation or cytotoxic drugs would therefore not be sufficiently selective.

Rhim and Brinster produced transgenic mice with a defective urokinase plasminogen activator controlled by the albumin promoter. The defective hepatocytes were replaced eventually with foreign hepatocytes, including transplanted rat cells³. Though this model establishes several principles important to the production of hybrid livers, it would not be practical as a cost-effective system. Many of the pups had hypofibrinogenemia and died of neonatal hemorrhage⁴. Most likely any system with congenital compromise of the liver would produce pigs that would be difficult to raise. A preferable system would produce pigs that are physiologically normal so that they can be readily bred and raised. The fetal hepatocytes could be selectively destroyed at the time that the transplant hepatocytes are injected.

Assuming that the liver contains multiple species-specific factors that support the pig, it may be necessary to preserve a portion of the pig liver until transplantation into the human recipient. It would therefore be desirable to control the degree of destruction of native hepatocytes.

If a hybrid human/pig liver could be produced, it would still be susceptible to immune rejection. Hepatocytes express relatively little histocompatibility antigen. Replacing pig hepatocytes with human hepatocytes, therefore, would have only a modest effect on reducing the antigen disparity. More significantly, the remaining pig endothelial cells would still be major targets for rejection.

System innovation for overcoming the hurdles

The above hurdles would be overcome by targeting the native pig hepatocytes with a suicide gene and prodrug. Suicide genes produce enzymes not normally present in the cells. The enzyme converts a non-toxic prodrug into a toxic substance. The best known system involves thymidine kinase (tk) and the prodrug ganciclovir. Tk, produced by Herpes viruses, is not normally present in uninfected cells. Herpes infected cells convert the ganciclovir and are destroyed. This system will be compared with a second system employing the gene for cytosine deaminase and the non-toxic prodrug 5-fluorocytosine. The prodrug is converted into the toxic 5-fluorouracil (5FU).

To prevent significant injury to other pig cells or the transplanted hepatocytes, the injury will be directed to the native pig hepatocytes. This can be accomplished by two distinct approaches. First, transgenic pigs will be produced that express the suicide gene in all cell lines. Immunoliposomes would be prepared containing the appropriate prodrug and carrying antibodies specific to antigens expressed on hepatocytes, such as asialoglycoprotein receptor. Immunoliposomes have been used for targeting tissues, such as delivery of cytotoxic agents to hepatomas. Liposomes containing ganciclovir have been used to treat retinal CMV infections. The second method would construct the vectors with an albumin promoter. The suicide gene would be expressed only within the hepatocytes. The prodrug would be administered systematically.

The advantage of the tissue specific expression of the suicide gene is that the prodrug could be given periodically to the sow. The prodrugs ganciclovir and 5-FC cross the placenta membrane⁵. The immunoliposomes would need to be administered into the fetal pig along with the transplant hepatocytes.

From 3/7/00
ATP/Gm

**Proliferation of Human Hepatocytes in Pig Spleens
And Detection of Human Liver Proteins**

<i>Piglet</i>	<i>Hepatocytes in the Spleen</i>	<i>Est. Expansion of Injected Hepatocytes*</i>	<i>Human Serum amyloid A#</i>	<i>Human Serum AAT#</i>
75-1	3.6x10 ⁷	18X	0.12%	0.9%
75-2	1.6x10 ⁸	90X	0.23%	22%
75-3	5.8x10 ⁷	29X	0.10%	5.5%
75-4	7.6x10 ⁷	38X	ND	ND
75-5	1.6x10 ⁸	80X	ND	ND
316-1	ND	--	0.90%	ND
316-2	ND	--	0.73%	ND
316-3	ND	--	0.24%	ND
361-1	ND	--	0.45%	ND
361-2	ND	--	0.52%	ND

*Number of splenic hepatocytes/Number of Injected Hepatocytes

Concentrations compared with concentration in human control sera.

The second and third sows produced six and eight live births. Serum analysis for SAA and alpha-1 antitrypsin were consistent with chimerism in two and three pigs respectively. These pigs have been followed for up to three months. At three months one of 2 pigs (316-2) shows persistent human proteins (0.6% SAA). That pig currently weighs 20 kg and is clinically normal and healthy. At 60 days of age, three of 4 evaluated pigs had human SAA in the serum.

The preliminary findings, therefore, support the proliferation and function of human hepatocytes in the fetal and newborn pigs. The injected hepatocytes expanded at least 18 to 90 fold and produced human proteins. The detection of human liver proteins in the serum confirms the presence of viable hepatocytes in the pigs. Furthermore, the localization to the spleen and the failure to identify hepatocytes in the liver is consistent with the hypothesis that the infused hepatocytes are competing with native hepatocytes.

Transfection of Porcine Renal Epithelial Cells.

By introducing a retroviral vector into oocytes rather than targeting embryos, Chan and Bremel were able to achieve a very high efficiency of transfection and avoid the problems of mosaicism⁸. The transfections were performed with bovine oocytes.

To determine the feasibility of this technology with porcine cells, a cell line of porcine renal tubular epithelial cells (PK-15) was utilized. The vector was a replication defective vector based on the Moloney murine leukemia virus, pseudotyped with the envelope glycoprotein of vesicular stomatitis virus. The vector included a neomycin phosphotransferase and a reporter gene (GFP, green fluorescent protein). The PK-15 cells were transfected with a high titer of the vector and the resistant cells selected. The cells were cultured for three weeks. They demonstrated diffuse expression of the GFP.

Prevention of Xenograft Rejection with Surrogate Tolerogenesis.

As an alternative to inducing immune unresponsiveness *within the recipient*, we propose instead to induce immune tolerance to the donor *within the xenograft donor*, and adoptively transfer the tolerance back to the recipient. We have termed this process "surrogate tolerogenesis"³⁸. The induction of tolerance within the donor animal provides considerably greater flexibility and opportunity. For example, with current approaches developmental tolerance could only be applied to fetal or newborn patients. With surrogate tolerogenesis, however, developmental tolerance can be established within fetal donor animals

PROJECT _____

Notebook No. _____

43

Continued From Page _____

11/9/00

CO

SFC crosses placenta - PDR

AFP

192 Lamp

found in brain up to 3rd month -

Serum AFP - fetal yrs higher -

than Alb. for at least 90 days

Alb promoter

PEE at -155 and needed for Es - Varchek '00

Licensing with BASF (i+TA)

Carl O'Brien 508-842-2500

FAX - 508-755-8361

- 1) What the field of use will be -
describe how used
- 2) non-exclusive or exclusive
- 3) Territory

11/13/00

Cmu promoter - Brenda Akers 319-335-4546

brenda-akers@uiowa.edu fax 319-335-4489

protein product license, intended use, contact info
(.5%)

Continued on Page

Read and Understood By

Signed

Date

Signed

Date

AFP

11/14/00

AFP mRNA in gut + kidney - Navel '92

Site 1b in MER1 binds liver, kidney, brain - Zhang '92

cell-spec expression due to protein - Liu '95

AFP mRNA in lymphocyte blastogenesis - Leclercq-Fassinet
(PHA-stimulated) 6x5 higher '89AFP specificity + repression due
to 1st kb upstream - upstream
enhancer elements are inactive in kidney -
also Rebeck '92Enhancer III very little activity
① > 2 > 3 - Zhang '92

Enhancer local

Transgenic mice AFP-TK - Macri '94

deletion of repression region allows expression -
Wern '91

Albumin mRNA not in fetal kidney - Rebeck '92

hereditary persistence AFP at -119 - McVey '93

Transgenic mice AFP - Liu '95

Similar interests of enhancer to regulate - Ishikawa

Continued on Page

199

Read and Understood By

Signed

Date

Signed

Date

GFP

11/13/00 called Court Tucker at 858-404-8416
(Clontech) fax 858-404-674
left message to call back w/ info on lica

11/14/00 talked to ? in Court's office -
would like company info - how large, # researchers
field of use
used commercially?
relationship w/ univ
this covers SOST mutations

11/15/00 Kozinski says EGFP
exit at 488 emission at ~
good for flow and could get f1E sets
for microscope

12/4/00 GFP may cause less damage than ~~excitation~~
at 513nm
instead of BFP(380) GFP(433)
EGFP(488)

12/10/00 faxed info to Eric at Court's office - will
respond after 11/1/01

Continued on Page _____

Read and Understood By _____

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Date _____

Signed _____

Date _____

Test

12/6/00 background level of expression
may need grounding by ~~ITS~~^{red} - French 11.4 89

Continued on Page _____

Read and Understood By _____

PROJECT _____

Continued From Page _____

TK

BVarnu, S-BVdu (pyrimidine nucleoside analogues)
much lower bystander effect

E-GCV highest SI - Degreus '99

11/30/00 BSE mediated by soluble factors in
DHD/K12 cells - Prince '99

Talked to Larry Shier 510-923-8290
he'll call back Steve Rosenberg - 3303

JP David Lentini 510-923-2706
dept, 2707

talked to Steve Harrison - he refers to
- 4037

Shawn O'Connell 510-923-3301 wants
patents #5

11/30/00 Talked to Michelle (InvivoGen)
they have fusion co/app + tk + EC4-1
she'll come up patent info

Continued on Page _____

Read and Understood By _____

Sinned _____

Date _____

C.O

11/13/00 S-Fc S-Fu permeate tumor cells by
passive diffusion - Erbs '00
QUEST - FCPI + FUR1 fusion -
high bystander but lower dose required

Sent e-mail to Erbs@transgen fr
- Erbs '00
Inquiry about licensing + samples

* S-Fc does - limiting toxicity by
bacteria in gut - Hansen '99

11/20/00 Candida albicans CO? - Andersen '00
called invovogen - possible license info

Scott called - they have patent
on fusion product, not sure about
just FCPI - he'll call back
'Cytosine deaminase patent' - Oncogen Peter Su

Continued on Page _____

Read and Understood By _____

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Date _____

AFP

11/14/00 silencer - needs @ trans factors - Nakabayashi '91
in hepatomas

AFP (E) / AFP (P) amplified - He '00 + '99

3rd month in pregnancy AFP disappears - Marcs '85
no mRNA assay from brain

200 bp ^{enhancer} conserved in species - Bernier '93

Rat AFP works in human cells - Gerolami '00

transgenic mice truncated AFP - Chebratians '95

rat AFP expression in liver of transgenic mice
w/ proximal enhancer - Cailliau '00

moved enhancers closer - Wen '93

11/15/00 EII higher in liver than yolk + gut

NO silencer region (3' UTR) ^{active} EII higher in yolk than liver - Remesh '95
EIII sensitive to integration S.E. -
+ function in CNS

more with TK
7.6 kb AFP promoter in transgenic mice
with HCC - Macri '94

mouse to +37
- 3800 AFP functional in fetal mouse - Chebratians '95

Rat - 3100 to +102 not functional in

Species specific? fetal ^{mouse} liver. - Cailliau '00

Signed

Date

Signed

Date

AFP

11/13 or 11/16 - yeth sac gone in 45-50 days -

Minced '80
Histochem
pig

11/17/00 alb enhancer 1.9 kb segment
junctions with in adult mice - Finck '87

11/20/00 Core lab in \$1600 to sequence

Invitrogen TA cloning kit recommended
for sequencing

Paul Frey - lambda library as backup

recommends plasmid tags for 12-20 kb
(Gibco)

11/21/00 Core lab up to 10-12 weeks to seq 10 kb

Continued on Page

Read and Understood By

Signed

Date

Signed

Date

Other Suicide

11/14/00 PNP (purine nucleoside phosphorylase) -
high burstable effect - Mohr '00

Plasmid info

11/28/00 Arpad Lanyi 9.8258
8261

11/22/00 drug selectable genes can influence
expression by LCR driven genes - even
when on another plasmid

11/30/00 Lyophilization - freeze drying to save

★ Subculturing can lead to change in
characteristics Hunter-Covera, Bell 196 Book
MCT-B+I

Cover of mineral oil, refrigerate
minimal nutrient

Continued on Page

13 74

Date

Signed

Date

IRES

11/28/03

Quica says lower expression in
(Quica)
(contacts) downstream of ECMV IRES
lower expression in attenuated II

~~PLP-IRESneo~~ = non-attenuated
PLP-IRES2-EGFP

She says

looks the same as pLP-IRESneo to me

CMV

1/10/04

Kappen states very few IRES are
up constitutive expression

I now think CMV not the best idea

Continued on Page _____

Read and Understood By _____

Technical Milestones:

No changes.

Technical Progress and Impact (November, December 2000):

1. Development of transgene constructs. The original proposal involved four constructs. Two constructs used thymidine kinase as the suicide gene. Two constructs used cytosine deaminase. One of each of these used the albumin promoter for specific expression in the liver. One of each used a CMV promoter, providing universal expression. Specific depletion of the hepatocytes would then be accomplished with immunoliposomes.

A critical review and analysis of the proposed genes and promoters led to refinement of the proposed constructs. Some of the issues are summarized here.

Thymidine kinase and male sterility? At the time of the proposal one group had observed male sterility with transgenic mice (Cohen et. al.). Since then we have heard of another group experiencing the same complication in a new set of transgenic mice using tk with an albumin promoter. This could be a fatal problem with our system. We intend to cross homozygous tk boars with wild type sows. Prodrug given to the sow would then deplete hepatocytes in the fetuses, but not the sow. Also, the herd can be expanded much more rapidly using semen from a homozygous boar. To resolve this problem, we have obtained the truncated form of tk, which is not associated with male sterility.

Albumin or alpha fetoprotein promoter for liver specific expression? For optimal utility, the suicide gene should be expressed in the liver during both fetal development and post-natal development. The initial depletion takes place at about 45 days gestation. It could be advantageous, however, to repeat the depletion of pig hepatocytes after the pig is born, or even after the hybrid liver is transplanted. In reviewing the ontogeny of albumin, however, albumin was found to be only minimally produced in the fetal pig. The alpha fetoprotein promoter was considered. Alpha fetoprotein, however, is expressed primarily in fetal development and not post-natal. AFP is also expressed in the yolk sac. This proved to not be a serious issue since the yolk sac is resorbed before 45 days gestation. The proposed constructs with albumin promoter will include AFP enhancers (but not the AFP promoter). The construct with an AFP promoter will have the silencer depleted, thought to be responsible for reduced expression post-natal. If the two constructs still lead to expression in only the adult or fetal pig, the transgenic pigs could be crossbred to contain both transgenes.

Bacteria or yeast cytosine deaminase? Most studies use the bacterial cd. There was concern, however, that the dose of 5-FU necessary to deplete the cells would also sterilize the colon of the sow, leading to problems in raising the pigs. The yeast cd, however, was found to be more than 100 times as sensitive as the bacterial cd. The gene has been ordered.

Toxicity of green fluorescent protein? The original proposed constructs would contain gfp as a reporter gene, easily detected with ultraviolet light. In addition to assisting with the development of transgenic pigs, the expression would provide an easy assay for determining chimerism within the hybrid liver. With the universal expression (CMV

promoter), the gfp could also assist with monitoring pig lymphocyte chimerism. The literature, however, has conflicting reports of possible toxicity of gfp. Though anecdotal, the development of transgenic gfp monkeys by Dr. A. Chan brought home the point. Two transgenic monkeys that produced gfp were stillborn. The only transgenic monkey to survive had the gene for gfp, as detected by PCR, but did not express the gfp. Consideration was made to have the gfp under the reverse tetracycline inducer. Expression would then be induced with an administration of tetracycline. The idea was abandoned, however, because of potential effects on the other genes and because of licensing and economic issues. We have therefore decided to exclude gfp from our constructs at this time. Dr. R. Prather has a herd of gfp pigs, which are being bred. If indicated, our transgenic pigs with the suicide genes could be bred with the gfp pigs.

Promoter for universal expression of transgene? Although the CMV promoter is generally used for universal expression of a transgene, there is increasing evidence that expression is not universal or uniform. We are currently exploring the ubiquitin promoter as an alternative.

Additional utility of transgenes in spillover technologies? Though our primary goal is to develop hybrid livers for xenotransplantation, that is considered a long-term goal. The system would be valuable, however, for spillover technologies which could provide near-term revenues. The growth of human hepatocytes or other human cells in pigs would be most useful for toxicology studies, animal models of human diseases, cost-effective production of new drugs, and the development of new vaccines.

To take advantages of these possibilities, two features have been included in the constructs. The first is the use of a universal promoter, providing expression in all cells. Specificity would be provided by immunoliposomes with tissue specific antibodies. This has been discussed previously. The second feature concerns *in vitro* purification of human cells taken from the hybrid pig. The same suicide genes used to create space for engraftment in the pig could be used to eliminate pig cells from a suspension of cells. To be most effective, however, it would be useful to have two suicide genes expressed, allowing for a double hit with prodrugs. The constructs with the universal promoter will also have two suicide genes (tk and cd) separated by an IRES gene. The two liver specific constructs will have either cd or tk. The best transgenic pigs from the two herds could then be crossbred.

The current design for the four constructs are as follows:

- a) Alb promoter-AFP enhancers-delta thymidine kinase
- b) AFP promoter (minus silencer)-yeast cytosine deaminase
- c) CMV promoter-delta thymidine kinase-IRES-yeast cytosine deaminase
- d) Ubiquitin promoter- delta thymidine kinase-IRES-yeast cytosine deaminase

The genes and sources are listed in the table:

Sequence	Source(s)
Albumin plus AFP enhancers	Currently being sequenced at Ximerex, Inc.
AFP minus first and third enhancer and silencing region	Currently being sequenced at Ximerex, Inc.
Truncated Thymidine Kinase	D. Klatzmann
Yeast Cytosine Deaminase (FCY1)	Invivogen
EMCV IRES	Clontech
CMV Promoter	Clontech

2. Development of Transgenic Pigs. The proposed project intended to produce transgenic pigs using perivitelline space injection of high titered retroviruses, a procedure developed by Gala Design, LLC. Recently the management of Gala Design requested that a license be negotiated before starting work on the project. Ximerex agreed with that suggestion. The issues have been defined and term sheets exchanged. We are optimistic that a license suitable to all will be negotiated shortly. In the event, however, that an agreement is not achieved soon, Ximerex has identified an alternative technology for developing the transgenic pigs.

Because the negotiations are taking longer than initially anticipated, Ximerex, Inc. has assumed responsibility for producing the constructs. To assist with this work, the Company recruited Dr. Carlos E. Sosa, M.D. Dr. Sosa has approximately 10 years experience with the molecular biology of viruses and producing constructs.

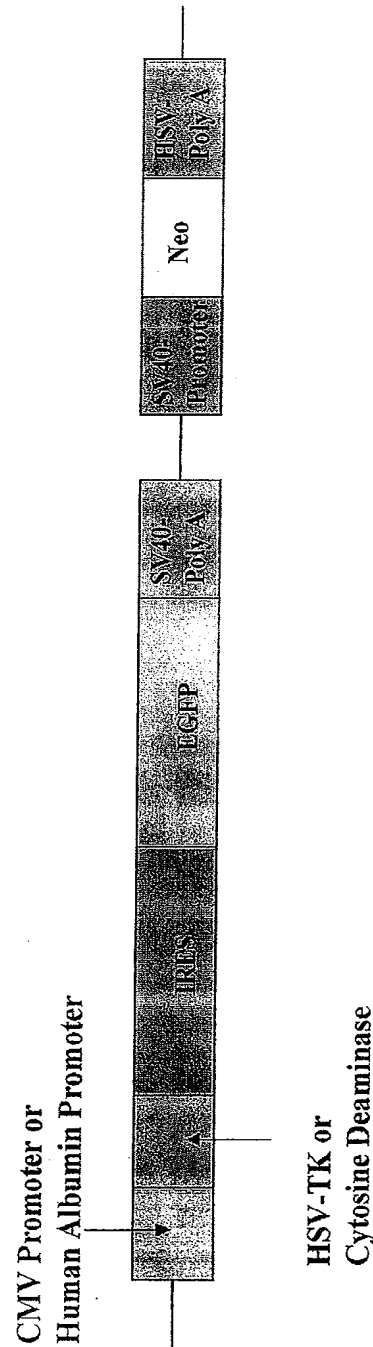
We have also discussed in detail work with Dr. Randall Prather, Professor of Animal Sciences at University of Missouri, Columbia. Dr. Prather has first hand experience in producing transgenic pigs using both the Gala technology and our alternate technology. Some of the pigs will be produced at the Univ. Missouri and some will be produced at the UNMC satellite facility in Oakland, Nebraska. IACUC approval for the changes has been applied for at both institutions.

At this time, it is believed that the changes in plans will not significantly affect our timetable or overall budget.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. Due to policy changes caused by a merger of the UNMC with a local hospital, the personnel and equipment previously used for fetal pig injections are no longer available. Alternative arrangements have been made.

One weakness of our technology was the inability to stain tissues for human cells. Immunoperoxidase stains have now been developed, which are specific and can be used with frozen and fixed tissue sections. We are now staining tissues collected from previous studies for cells producing human albumin.

Transgene Construct(s) Designed to Demonstrate Expression of Suicide Gene(s) and Green Fluorescent Protein Expression in Liver and Control Tissues



Separate constructs with the Albumin promoter driving Herpes Simplex Virus Thymidine Kinase (HSV-TK) or Cytosine Deaminase and Green Fluorescent Protein (EGFP) will be prepared for microinjection. The CMV promoter may also be used for early studies and as a control.

*From ATP Oral Presentation
(Powerpoint) July 13, 2000*

July 5, 2000

Gordon Todd III, Ph.D.
David A. Crouse, Ph.D.
Co-Chairs, IACUC
3022 Eppley Science Hall
University of Nebraska Medical Center
600 South 42nd Street
Omaha, Nebraska 68198-6810

Re: New Protocol entitled Human/Pig Hybrid Livers for Transplantation

Dear Drs. Todd and Crouse:

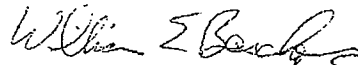
Enclosed is a protocol for the first year of an anticipated three year program. We just recently received notice that we are a semifinalist for our ATP proposal and will need to present the final materials on July 14. If an expedited review could be completed before then, it would help greatly in obtaining funding for the program. I apologize for not being able to give you more time.

We believe that the procedures qualify as a category C protocol. However, the fetal pig injections and Cesarian sections use the same procedures that were approved for protocol 98-012-04, Surrogate Tolerogenesis in Xenotransplantation. The evaluation of transgenic pigs are performed after euthanasia. The transgenic mice receive an i.p. injection of prodrug or immunoliposomes and are euthanized three days later.

Although recombinant DNA was checked as a possible biohazardous material, in fact we are only working with transgenic animals. The use of the DNA is performed at the Transgenic Mouse facility at UNMC and covered by their umbrella protocol (J.M. Salbaum, Ph.D.). The transgenic pigs would be produced at the University of Illinois (Urbana, IL, Matthew Wheeler, Ph.D.)

Thank you for your help and for your patience.

Sincerely,



William E. Beschoner, M.D.
Professor of Surgery
Transplantation, UNMC

Encl.

November 15, 2000

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum

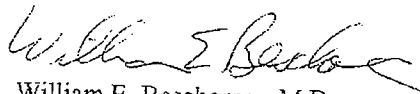
This letter of intent is to inform you our interest in recruiting your assistance in developing transgenic mice. These mice would be used for developing technology leading to the engraftment of human cells in transgenic pigs.

We plan to provide you with three constructs. Each construct would contain green fluorescent protein as a reporter gene and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the transgenic mice is 00-0094.

I hope that your laboratory can help us with this project. We look forward to working with you.

Sincerely,



William E. Beschorner, M.D.
Professor of Surgery, UNMC

5. Mouse Model of Hepatocyte Depletion. As indicated previously, arrangements have been made with the core transgenic mouse facility of the UNMC. The constructs for the transgenic mice are being prepared (see above).

We discussed the possibility of using transgenic mice produced by Eric Sandgren (U. Wisc.) These mice express thymidine kinase under the albumin promoter. Although Dr. Sandgren was agreeable to collaboration, two problems were identified. First, his colony currently has mouse hepatitis. Secondly, they had male sterility, which would make it difficult to do the fetal studies.

Summary of Project Changes:

Matthew Wheeler, from the University of Illinois at Urbana-Champaign, is no longer available to work with us. As a contingency plan, we are establishing collaboration with Randall Prather, Ph.D. at the University of Missouri at Columbia.

Problems and Opportunities:

President and Chief Scientific Officer, William E. Beschoner, has resigned his appointment from the University of Nebraska Medical Center to avoid a potential conflict of interest with this subcontractor.

Business Issues:

Only one piece of additional information that was not included in our Quarterly Business Report: We are currently negotiating license and use agreement with Gala Design for their technologies.

Upcoming Meetings:

None Scheduled

From ATP Quarterly Report
6 11/00 + 12/00

March 20, 2001

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum:

This letter is to serve as an agreement between our laboratories regarding the development of transgenic mice. If these terms are acceptable with you, please sign and return the original letter.

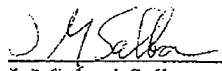
It is agreed that your laboratory will make a best effort at producing transgenic mice, including transfection of up to 500 mouse embryos per construct. For each construct, Ximerex, Inc. will reimburse the university \$2300 plus indirect costs (approximately 46%). The first construct should be ready by the end of April 2001.

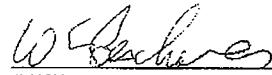
We plan to provide you with four constructs. Each construct would contain a promoter and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the transgenic mice is 00-0094.

We very much appreciate your advice and help with our project to produce hybrid pig organs. As discussed, we are setting up a transgenic facility to produce pigs, based in large part on advice from you and Judy Stribley. In the future, if we can help your laboratory in any way, i.e. use of equipment, reagents, technology, etc., we would be most pleased to do so.

Agreed:


J. Michael Salbaum, Ph.D. 3/26/01
Director, Transgenic Core Laboratory
Center for Human Genetics


William E. Beschoner, M.D. 3/20/01
Adj. Professor of Surgery, UNMC
President and CSO, Ximerex, Inc.

PROJECT

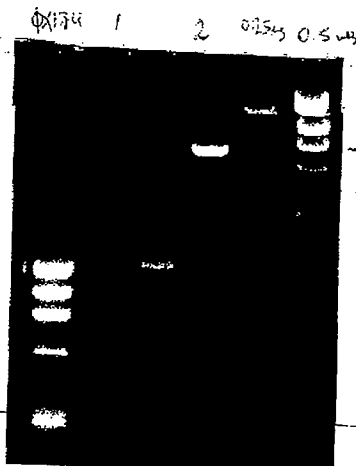
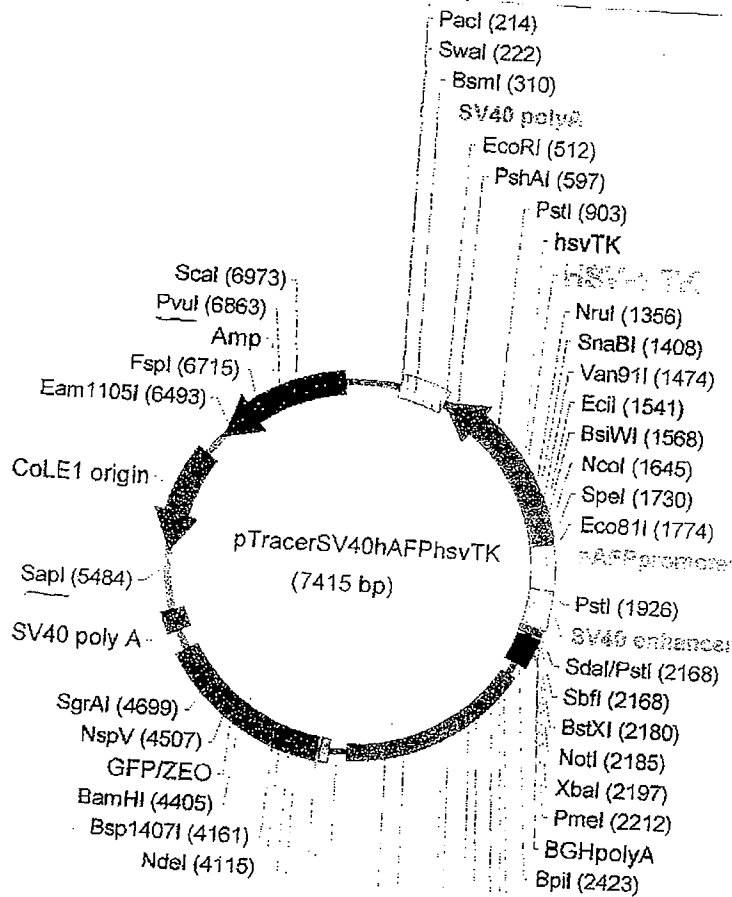
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Transgenic Mice

8-18-01

Fragment sent to Mike Sedham

8-18-01



1 = pTracer pA1b ΔTK cut with PvuI and

Sap I has fragment (6036)

2 = pTracer pA1 ΔTK PvuI/Sap I

Deleting part of an origin

gene and origin of replication

(ColE1 origin) 1356 bp

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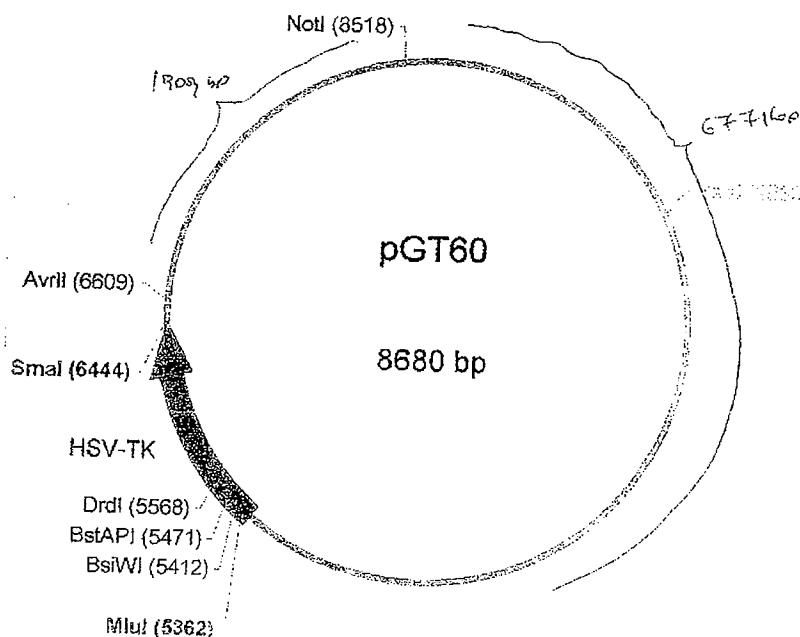
Date

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Date

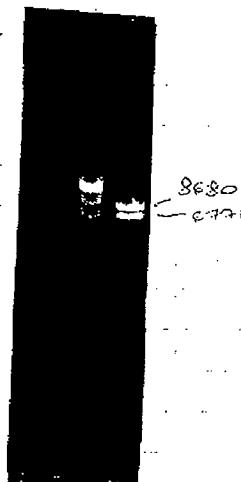
9-18-01

Preparing pGT60TK for transgenic mice
 Digestion with NotI + AvrII



1% agarose

Some
DNA
was not
seen
cut by
the
AvrII
enzyme
(2nd
lane)



0.8% agarose

pGT60 & TK #15
 was then sent for
 transgenic mice
 ~ 10 mg of DNA

Sequence confirm
 the mutations
 on the two
 ATG sites

We have set up this system by generating a stably transfected cell line. PK-15, a pig epithelial cell line has been transfected with pGT60 Fcy/xTK. After 2 weeks in selection media containing hygromycin B, resistant cell colonies were lifted using glass rings and trypsin, and sub-plated in 24 well plates. After the cells grew to confluence, they were plated in 6 well and then in 100 mm plates.

We set up our prodrug-killing assay by using this pGT60 Fcy/xTK PK-15 stably transfected cell lines. Six well plates containing 70 percent confluent PK15 transfected cells were added 4mM ganciclovir in the culture media. Control cells were given media alone. Cells were monitored for death. After 72 hours, cells with ganciclovir showed 60% mortality. The control cultures without ganciclovir had less than 10% mortality. At 96 hours 90% of the cells were dead. Cell mortality was measured by Trypan blue exclusion staining.

2. Development of Transgenic Pigs. As detailed in our letter to Dr. Chapekar, July 12, 2001, the best technology for producing transgenic pigs is nuclear transfer (cloning) using transfected fetal pig fibroblasts. This technology is relatively efficient, and avoids mosaicism.

3. Development of Immunoliposomes. Dr. Joshi's laboratory has produced the first liposomes. They are 100-200 nm in diameter and relatively stable. Antibodies have been ordered to produce immunoliposomes.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Sixteen litters of pigs have been infused with human hepatocytes or bone marrow. The hepatocytes were provided by Dr. Strom, Univ. Pittsburgh. They were fresh (not frozen) and came from two human donors. The bone marrow came from 5 human volunteers. Eleven of the sixteen litters have or will go to term. Six litters have been delivered. Some of the pigs were lost shortly after delivery. The tissues and serum are being evaluated for human hepatocytes and human liver proteins. The next group of five litters will be delivered shortly.

5. Mouse Model of Hepatocyte Depletion. The above three constructs have been provided to the UNMC transgenic mouse facility. An outbreak of mouse hepatitis forced a postponement of the production of transgenic mice. It is expected to resume about August 1, 2001. This subproject is still on schedule.

Summary of Project Changes:

As described above, we intend to produce transgenic pigs using nuclear transfer technology. The nuclei in pig oocytes will be replaced with nuclei from transfected fetal fibroblasts. The oocytes are activated and implanted into the surrogate gilts.

Problems and Opportunities:

There were no significant new problems or opportunities developing this quarter.

*From ATP Quarterly Report
in 4/01 - 6/01*

Ex 32

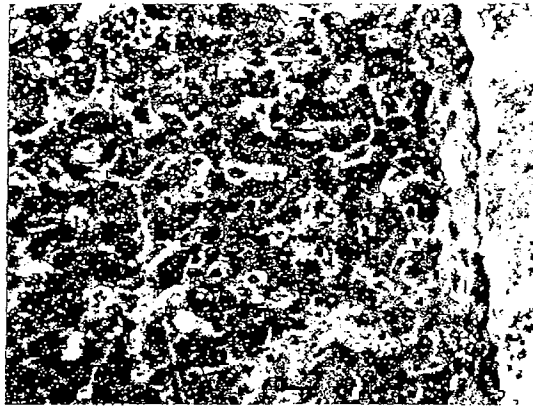


Figure 7. High power view of porcine liver stained for human albumin. The upper 75% of the hepatocytes are producing human albumin. The architecture is normal.

5. Mouse Model of Hepatocyte Depletion. Constructs containing the albumin promoter and thymidine kinsase (Construct A, above) and containing the universal promoter and both suicide genes (Construct C, above) were linearized and provided to the transgenic mouse core laboratory. The DNA was injected into mouse zygotes. Recently 150 pups were born. They are currently being screened by PCR for the transgene.

From ATP Quarterly Rpt
7/01 - 10/01

Using the same strategy previously mentioned, we transfected the plasmid pGT60xTK/FCy. this construct contains two suicide genes (xTK and cytosine deaminase) controlled by very powerful universal or ubiquitous promoters. The selectable antibiotic that we used in this case is hygromycin B, 200 ug/ml.

These cell lines do not contain GFP so the possibility of visualizing them using the fluorescence microscope was not available.

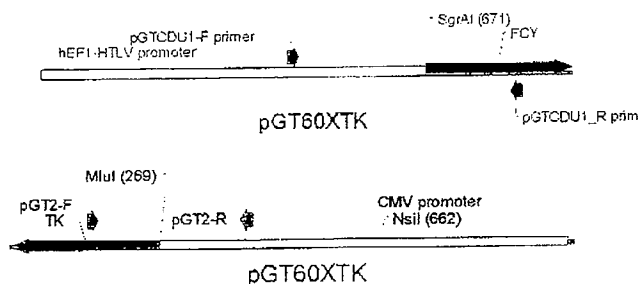


Figure 2. pGT60xTK/FCY showing the two pairs of primers for the promoter suicide gene junctions of xTK and FCY genes.

For this construct we are in the process of selecting cell lines and screening by PCR.

2. Development of Transgenic Pigs. A license has been negotiated with the intellectual property office of University of Missouri. Transgenic pigs would be produced using nuclear transfer technology, as developed by Dr. Randall Prather (US #6,211,429, Complete oocyte activation using an oocyte-modifying agent and a reducing agent). The legal departments of Ximerex and the University of Missouri are reviewing the agreement.

Scott Thompson continues to develop the nuclear transfer technology at Ximerex, inserting fetal pig fibroblasts into enucleated pig oocytes. It is anticipated that implantations into surrogate sows will begin in February 2002.

3. Development of Immunoliposomes. Dr. Shantaram Joshi is enhancing the immunoliposome technology, optimizing the sonication methods used.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Fetal pigs (31 to 56 days) were infused with human cord blood. After they are born, they will be analyzed for evidence of human liver proteins in the serum and hepatocytes in the tissues.

5. Mouse Model of Hepatocyte Depletion.

In order to produce transgenic mice, vector containing pTracerpAlbxTK were cut using restriction enzymes so that areas not essential to the function of the transgene were eliminated.

*From ATP Quinberg
Report for 10/01-12/01*

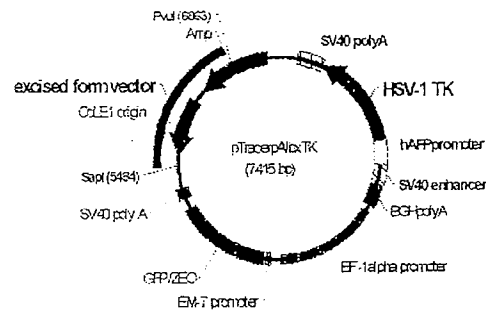


Figure 3. Non-essential areas for transgene function were excised.

Using the same approach, pGT60xTK/Fcy was also cut with appropriate restriction enzymes (Fig 4).

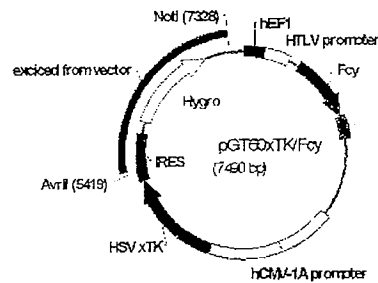


Figure 4. Non-essential areas for transgene function were excised.

Twenty micrograms of highly purified, linearized DNA was sent to the UNMC transgenic core facility for transgenic mouse production.

Sixty eight pups were born from pTracerpAlbTK. And 92 were born from the mice injected with pGT60xTK/Fcy transgene. These pups can potentially have the aforementioned plasmid DNA integrated into their genome. In order to screen these animals, their tails were clipped and DNA was extracted. A genomic PCR has been established using primers that span the promoter area and the beginning of the suicide gene. Using this PCR approach we have tested 160 DNA samples from the mouse-tails. Fig 5. shows a typical PCR gel. Lane number 8 shows a positive amplicon of 230 bp. M= molecular weight marker



Figure 5. Agarose gel stained with ethidium bromide and exposed to UV light.

We have obtained ten founder mice with these two constructs, see table 2. We are in the process of breeding these founders with male or female FVB normal mice to generate the first generation of transgenic mice.

	Number of Pups	Number of positives	% positives	Males	Females
pTracerpAlbx TK	68	5	7.35	3	2
PGT60Fcy	92	5	5.43	2	3

Table 2. Characteristics of transgenic mice.

Summary of Project Changes:

There were no new developments in this quarter.

Problems and Opportunities:

There were no new problems or opportunities this quarter.

Business Issues:

There were no new developments this quarter.

Upcoming Meetings:

No meetings were scheduled.

Technical Milestones:

No changes.

Technical Progress and Impact (November, December 2000):

1. Development of transgene constructs. The original proposal involved four constructs. Two constructs used thymidine kinase as the suicide gene. Two constructs used cytosine deaminase. One of each of these used the albumin promoter for specific expression in the liver. One of each used a CMV promoter, providing universal expression. Specific depletion of the hepatocytes would then be accomplished with immunoliposomes.

A critical review and analysis of the proposed genes and promoters led to refinement of the proposed constructs. Some of the issues are summarized here.

Thymidine kinase and male sterility? At the time of the proposal one group had observed male sterility with transgenic mice (Cohen et. al.). Since then we have heard of another group experiencing the same complication in a new set of transgenic mice using tk with an albumin promoter. This could be a fatal problem with our system. We intend to cross homozygous tk boars with wild type sows. Prodrug given to the sow would then deplete hepatocytes in the fetuses, but not the sow. Also, the herd can be expanded much more rapidly using semen from a homozygous boar. To resolve this problem, we have obtained the truncated form of tk, which is not associated with male sterility.

Albumin or alpha fetoprotein promoter for liver specific expression? For optimal utility, the suicide gene should be expressed in the liver during both fetal development and post-natal development. The initial depletion takes place at about 45 days gestation. It could be advantageous, however, to repeat the depletion of pig hepatocytes after the pig is born, or even after the hybrid liver is transplanted. In reviewing the ontogeny of albumin, however, albumin was found to be only minimally produced in the fetal pig. The alpha fetoprotein promoter was considered. Alpha fetoprotein, however, is expressed primarily in fetal development and not post-natal. AFP is also expressed in the yolk sac. This proved to not be a serious issue since the yolk sac is resorbed before 45 days gestation. The proposed constructs with albumin promoter will include AFP enhancers (but not the AFP promoter). The construct with an AFP promoter will have the silencer depleted, thought to be responsible for reduced expression post-natal. If the two constructs still lead to expression in only the adult or fetal pig, the transgenic pigs could be crossbred to contain both transgenes.

Bacteria or yeast cytosine deaminase? Most studies use the bacterial cd. There was concern, however, that the dose of 5-FU necessary to deplete the cells would also sterilize the colon of the sow, leading to problems in raising the pigs. The yeast cd, however, was found to be more than 100 times as sensitive as the bacterial cd. The gene has been ordered.

Toxicity of green fluorescent protein? The original proposed constructs would contain gfp as a reporter gene, easily detected with ultraviolet light. In addition to assisting with the development of transgenic pigs, the expression would provide an easy assay for determining chimerism within the hybrid liver. With the universal expression (CMV

From ATP Quarterly
Progress Report for Nov, Dec 2000

promoter), the gfp could also assist with monitoring pig lymphocyte chimerism. The literature, however, has conflicting reports of possible toxicity of gfp. Though anecdotal, the development of transgenic gfp monkeys by Dr. A. Chan brought home the point. Two transgenic monkeys that produced gfp were stillborn. The only transgenic monkey to survive had the gene for gfp, as detected by PCR, but did not express the gfp. Consideration was made to have the gfp under the reverse tetracycline inducer. Expression would then be induced with an administration of tetracycline. The idea was abandoned, however, because of potential effects on the other genes and because of licensing and economic issues. We have therefore decided to exclude gfp from our constructs at this time. Dr. R. Prather has a herd of gfp pigs, which are being bred. If indicated, our transgenic pigs with the suicide genes could be bred with the gfp pigs.

Promoter for universal expression of transgene? Although the CMV promoter is generally used for universal expression of a transgene, there is increasing evidence that expression is not universal or uniform. We are currently exploring the ubiquitin promoter as an alternative.

Additional utility of transgenes in spillover technologies? Though our primary goal is to develop hybrid livers for xenotransplantation, that is considered a long-term goal. The system would be valuable, however, for spillover technologies which could provide near-term revenues. The growth of human hepatocytes or other human cells in pigs would be most useful for toxicology studies, animal models of human diseases, cost-effective production of new drugs, and the development of new vaccines.

To take advantages of these possibilities, two features have been included in the constructs. The first is the use of a universal promoter, providing expression in all cells. Specificity would be provided by immunoliposomes with tissue specific antibodies. This has been discussed previously. The second feature concerns *in vitro* purification of human cells taken from the hybrid pig. The same suicide genes used to create space for engraftment in the pig could be used to eliminate pig cells from a suspension of cells. To be most effective, however, it would be useful to have two suicide genes expressed, allowing for a double hit with prodrugs. The constructs with the universal promoter will also have two suicide genes (tk and cd) separated by an IRES gene. The two liver specific constructs will have either cd or tk. The best transgenic pigs from the two herds could then be crossbred.

The current design for the four constructs are as follows:

- a) Alb promoter-AFP enhancers-delta thymidine kinase
- b) AFP promoter (minus silencer)-yeast cytosine deaminase
- c) CMV promoter-delta thymidine kinase-IRES-yeast cytosine deaminase
- d) Ubiquitin promoter- delta thymidine kinase-IRES-yeast cytosine deaminase

The genes and sources are listed in the table:

Sequence	Source(s)
Albumin plus AFP enhancers	Currently being sequenced at Ximerex, Inc.
AFP minus first and third enhancer and silencing region	Currently being sequenced at Ximerex, Inc.
Truncated Thymidine Kinase	D. Klatzmann
Yeast Cytosine Deaminase (FCY1)	Invivogen
EMCV IRES	Clontech
CMV Promoter	Clontech

2. Development of Transgenic Pigs. The proposed project intended to produce transgenic pigs using perivitelline space injection of high titered retroviruses, a procedure developed by Gala Design, LLC. Recently the management of Gala Design requested that a license be negotiated before starting work on the project. Ximerex agreed with that suggestion. The issues have been defined and term sheets exchanged. We are optimistic that a license suitable to all will be negotiated shortly. In the event, however, that an agreement is not achieved soon, Ximerex has identified an alternative technology for developing the transgenic pigs.

Because the negotiations are taking longer than initially anticipated, Ximerex, Inc. has assumed responsibility for producing the constructs. To assist with this work, the Company recruited Dr. Carlos E. Sosa, M.D. Dr. Sosa has approximately 10 years experience with the molecular biology of viruses and producing constructs.

We have also discussed in detail work with Dr. Randall Prather, Professor of Animal Sciences at University of Missouri, Columbia. Dr. Prather has first hand experience in producing transgenic pigs using both the Gala technology and our alternate technology. Some of the pigs will be produced at the Univ. Missouri and some will be produced at the UNMC satellite facility in Oakland, Nebraska. IACUC approval for the changes has been applied for at both institutions.

At this time, it is believed that the changes in plans will not significantly affect our timetable or overall budget.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. Due to policy changes caused by a merger of the UNMC with a local hospital, the personnel and equipment previously used for fetal pig injections are no longer available. Alternative arrangements have been made.

One weakness of our technology was the inability to stain tissues for human cells. Immunoperoxidase stains have now been developed, which are specific and can be used with frozen and fixed tissue sections. We are now staining tissues collected from previous studies for cells producing human albumin.

With these sequences, restriction enzyme sites were designed to flank the promoter sequence. Specifically, Sda I will be placed about 50 bp upstream of a known DEIII transcription factor binding site. Nco I will be placed at the end of the pig promoter UTR (untranslated region).

In a similar fashion, the porcine alpha fetoprotein promoter has been identified and expanded. The sequence is being determined.

We are currently isolating the porcine ubiquitin promoter.

The suicide genes consist of delta thymidine kinase (tk) and yeast cytosine deaminase (fcy1). The delta tk plasmid was obtained from Dr. Klatzmann¹. Unfortunately, sequence analysis failed to identify the gene. We are currently producing our own truncated gene from the wild Herpes simplex virus I (HSV-1) thymidine kinase.

The fcy1 gene has been obtained from Invivogen and the sequence confirmed.

Synthesis of the constructs.

The construct consisting of AFP and fcy1 (construct b above) has been produced using the human AFP promoter. The pDriveAfp plasmid, containing human afp and SV40 enhancer was used as the backbone. The plasmid contains a Zeocin resistance gene. The yeast cytosine deaminase gene was inserted in-frame into pDrive, creating pDrive Fcy (2540 bp). The construct is illustrated in figure 2.

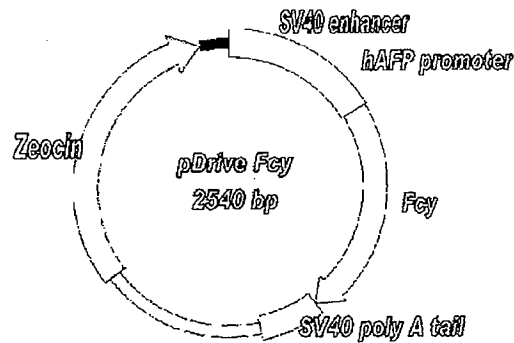


Figure 2. pDrive Fcy construct

This construct will be used for cell lines and transgenic mice. Prior to production of transgenic pigs, the human Afp promoter will be replaced with the porcine counterpart.

Using the same strategy, the HSV-1 TK gene was cloned behind the hAFP promoter, producing the pDrive HSV-1 TK (3200 bp, figure 3). The HSV-1 tk was subcloned from the commercially available pOrf HSV-1 TK (InvivoGen).

*From ATP Quarterly Report
12/01 Dec 2000 uSB
01/01-03/01*

EXHIBIT 35

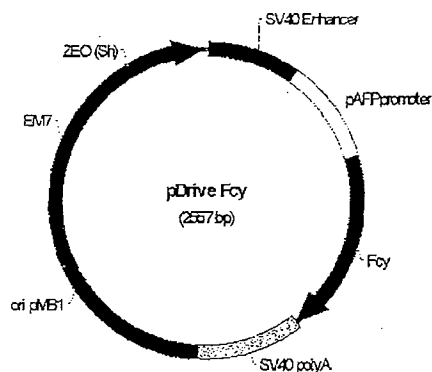


Figure 2

b. pDrive xTK

This construct contains the SV40 enhancer and the porcine albumin promoter that controls expression of a mutated version of the Herpes Simplex virus (HSV) thymidine kinase (xTK) gene (Fig 3). The gene was mutated by two rounds of site directed mutagenesis, resulting in the replacement of adenosine for cytosine at nucleotides 138 and 180. The nucleotide replacements resulted in a codon change such that a leucine replaces a methionine. These changes do not affect the enzymatic activity of the gene, since the catalytic pocket is located far away from the mutations. Expression of the TK gene in the testis would result in the infertility of the founder males, with catastrophic economical problems. The two codon changes prevent the ectopic expression of the TK gene in testis⁴.

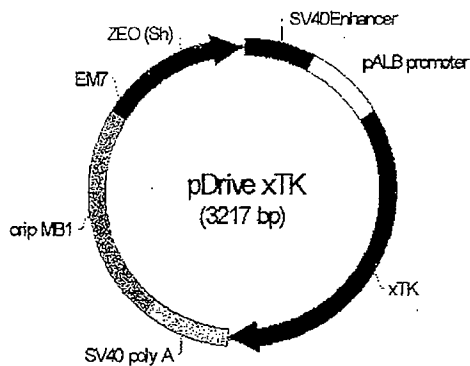


Figure 3

From ATP C. M. L. by ATP
04/01-06/01

July 5, 2000

Gordon Todd III, Ph.D.
David A. Crouse, Ph.D.
Co-Chairs, IACUC
3022 Eppley Science Hall
University of Nebraska Medical Center
600 South 42nd Street
Omaha, Nebraska 68198-6810

Re: New Protocol entitled Human/Pig Hybrid Livers for Transplantation

Dear Drs. Todd and Crouse:

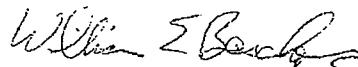
Enclosed is a protocol for the first year of an anticipated three year program. We just recently received notice that we are a semifinalist for our ATP proposal and will need to present the final materials on July 14. If an expedited review could be completed before then, it would help greatly in obtaining funding for the program. I apologize for not being able to give you more time.

We believe that the procedures qualify as a category C protocol. However, the fetal pig injections and Cesarian sections use the same procedures that were approved for protocol 98-012-04, Surrogate Tolerogenesis in Xenotransplantation. The evaluation of transgenic pigs are performed after euthanasia. The transgenic mice receive an i.p. injection of prodrug or immunoliposomes and are euthanized three days later.

Although recombinant DNA was checked as a possible biohazardous material, in fact we are only working with transgenic animals. The use of the DNA is performed at the Transgenic Mouse facility at UNMC and covered by their umbrella protocol (J.M. Salbaum, Ph.D.). The transgenic pigs would be produced at the University of Illinois (Urbana, IL, Matthew Wheeler, Ph.D.)

Thank you for your help and for your patience.

Sincerely,



William E. Beschoner, M.D.
Professor of Surgery
Transplantation, UNMC

Encl.

November 15, 2000

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum

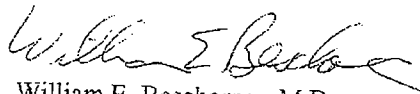
This letter of intent is to inform you our interest in recruiting your assistance in developing transgenic mice. These mice would be used for developing technology leading to the engraftment of human cells in transgenic pigs.

We plan to provide you with three constructs. Each construct would contain green fluorescent protein as a reporter gene and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the transgenic mice is 00-0094.

I hope that your laboratory can help us with this project. We look forward to working with you.

Sincerely,



William E. Beschorner, M.D.
Professor of Surgery, UNMC

5. Mouse Model of Hepatocyte Depletion. As indicated previously, arrangements have been made with the core transgenic mouse facility of the UNMC. The constructs for the transgenic mice are being prepared (see above).

We discussed the possibility of using transgenic mice produced by Eric Sandgren (U. Wisc.) These mice express thymidine kinase under the albumin promoter. Although Dr. Sandgren was agreeable to collaboration, two problems were identified. First, his colony currently has mouse hepatitis. Secondly, they had male sterility, which would make it difficult to do the fetal studies.

Summary of Project Changes:

Matthew Wheeler, from the University of Illinois at Urbana-Champaign, is no longer available to work with us. As a contingency plan, we are establishing collaboration with Randall Prather, Ph.D. at the University of Missouri at Columbia.

Problems and Opportunities:

President and Chief Scientific Officer, William E. Beschoner, has resigned his appointment from the University of Nebraska Medical Center to avoid a potential conflict of interest with this subcontractor.

Business Issues:

Only one piece of additional information that was not included in our Quarterly Business Report: We are currently negotiating license and use agreement with Gala Design for their technologies.

Upcoming Meetings:

None Scheduled

From ATP Quarterly Report
6 11/00 + 12/00

March 20, 2001

Reference: Transgenic mice for Hybrid Liver Project

J. Michael Salbaum, Ph.D.
Director, Transgenic Core Laboratory
Center for Human Genetics
Monroe Meyer Institute
986395 Nebraska Medical Center
Omaha, Nebraska 68198-6395

Dear Dr. Salbaum:

This letter is to serve as an agreement between our laboratories regarding the development of transgenic mice. If these terms are acceptable with you, please sign and return the original letter.

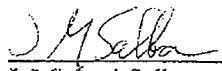
It is agreed that your laboratory will make a best effort at producing transgenic mice, including transfection of up to 500 mouse embryos per construct. For each construct, Ximerex, Inc. will reimburse the university \$2300 plus indirect costs (approximately 46%). The first construct should be ready by the end of April 2001.

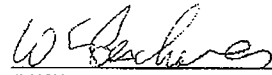
We plan to provide you with four constructs. Each construct would contain a promoter and a suicide gene. Our studies would require homozygous male mice. The background strain would be FVB.

The approved IACUC protocol that would accept the transgenic mice is 00-0094.

We very much appreciate your advice and help with our project to produce hybrid pig organs. As discussed, we are setting up a transgenic facility to produce pigs, based in large part on advice from you and Judy Stribley. In the future, if we can help your laboratory in any way, i.e. use of equipment, reagents, technology, etc., we would be most pleased to do so.

Agreed:


J. Michael Salbaum, Ph.D. 3/26/01
Director, Transgenic Core Laboratory
Center for Human Genetics


William E. Beschoner, M.D. 3/20/01
Adj. Professor of Surgery, UNMC
President and CSO, Ximerex, Inc.

PROJECT

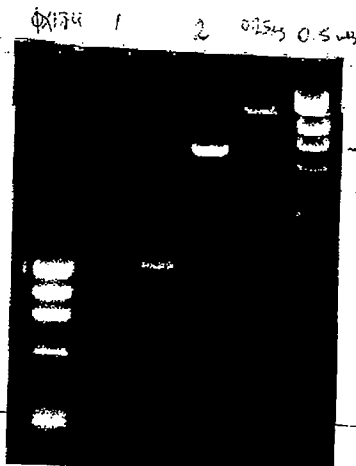
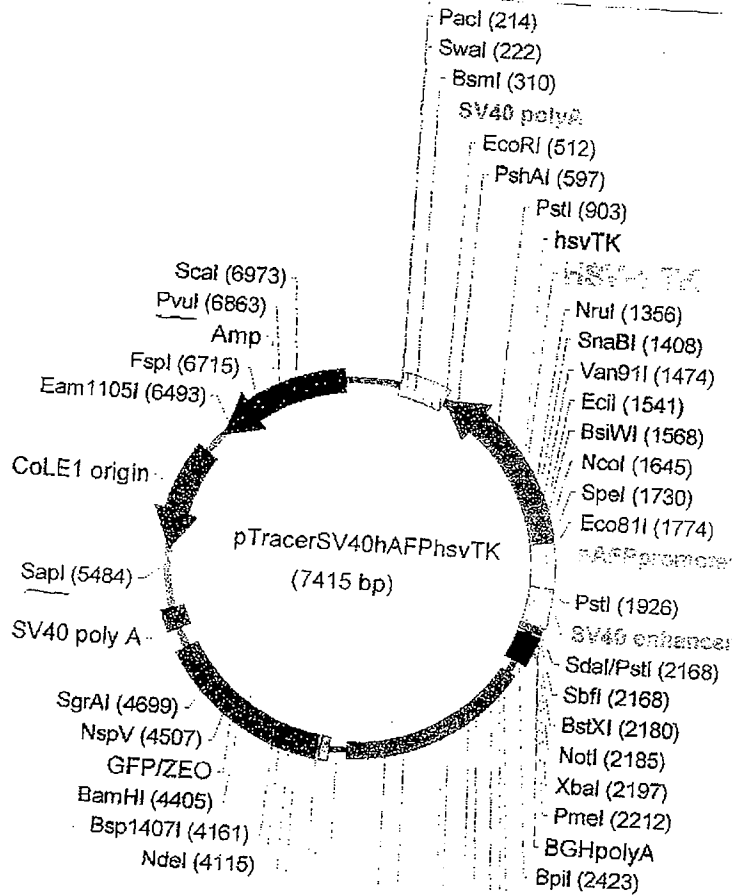
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Transgenic Mice

8-18-01

Fragment sent to Mike Sedham

8-18-01



1 = pTracer pALB ΔTK cut with PvuI and

Sap I has fragment (6036)

2 = pTracer pAL ΔTK PvuI/Sap I

Deleting part of an origin

gene and origin of replication
(ColE1 origin) 1356 bp

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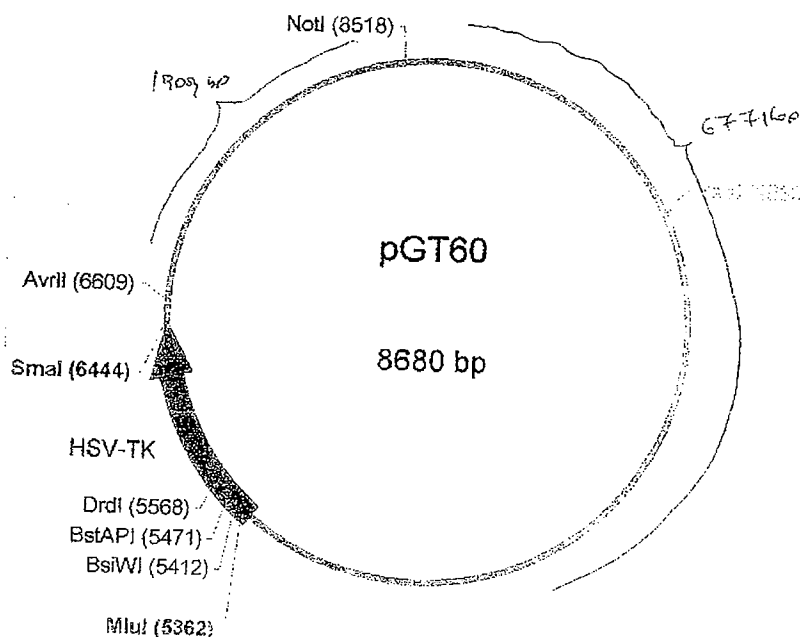
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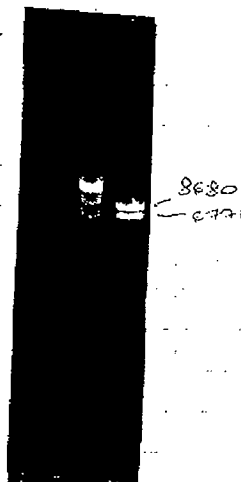
9-18-01

Preparing pGT60TK for transgenic mice
 Digestion with NotI + AvrII



1% agarose

Same
DNA
was not
seen
cut by
the
AvrII
enzyme
(2nd
lane)



0.8% agarose

pGT60 & TK #15
 was then sent for
 transgenic mice
 ~ 10 mg of DNA

Sequence confirm
 the mutations
 on the two
 ATG sites

We have set up this system by generating a stably transfected cell line. PK-15, a pig epithelial cell line has been transfected with pGT60 Fcy/xTK. After 2 weeks in selection media containing hygromycin B, resistant cell colonies were lifted using glass rings and trypsin, and sub-plated in 24 well plates. After the cells grew to confluence, they were plated in 6 well and then in 100 mm plates.

We set up our prodrug-killing assay by using this pGT60 Fcy/xTK PK-15 stably transfected cell lines. Six well plates containing 70 percent confluent PK15 transfected cells were added 4mM ganciclovir in the culture media. Control cells were given media alone. Cells were monitored for death. After 72 hours, cells with ganciclovir showed 60% mortality. The control cultures without ganciclovir had less than 10% mortality. At 96 hours 90% of the cells were dead. Cell mortality was measured by Trypan blue exclusion staining.

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5. Mouse Model of Hepatocyte Depletion. The above three constructs have been provided to the UNMC transgenic mouse facility. An outbreak of mouse hepatitis forced a postponement of the production of transgenic mice. It is expected to resume about August 1, 2001. This subproject is still on schedule.

Summary of Project Changes:

As described above, we intend to produce transgenic pigs using nuclear transfer technology. The nuclei in pig oocytes will be replaced with nuclei from transfected fetal fibroblasts. The oocytes are activated and implanted into the surrogate gilts.

Problems and Opportunities:

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*From ATP Quarterly Report
in 4/01 - 6/01*

Ex 32

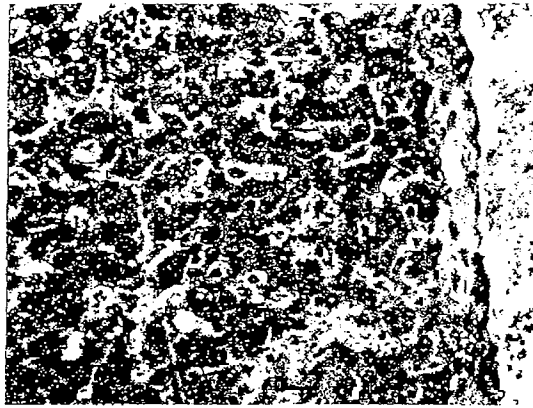


Figure 7. High power view of porcine liver stained for human albumin. The upper 75% of the hepatocytes are producing human albumin. The architecture is normal.

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From ATP Quarterly Rpt
7/01 - 10/01

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These cell lines do not contain GFP so the possibility of visualizing them using the fluorescence microscope was not available.

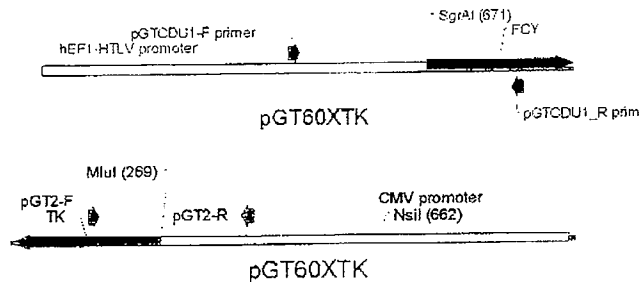


Figure 2. pGT60xTK/FCY showing the two pairs of primers for the promoter suicide gene junctions of xTK and FCY genes.

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Scott Thompson continues to develop the nuclear transfer technology at Ximerex, inserting fetal pig fibroblasts into enucleated pig oocytes. It is anticipated that implantations into surrogate sows will begin in February 2002.

3. Development of Immunoliposomes. Dr. Shantaram Joshi is enhancing the immunoliposome technology, optimizing the sonication methods used.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Fetal pigs (31 to 56 days) were infused with human cord blood. After they are born, they will be analyzed for evidence of human liver proteins in the serum and hepatocytes in the tissues.

5. Mouse Model of Hepatocyte Depletion.

In order to produce transgenic mice, vector containing pTracerpAlbxTK were cut using restriction enzymes so that areas not essential to the function of the transgene were eliminated.

*From ATP Quinberg
Report for 10/01-12/01*

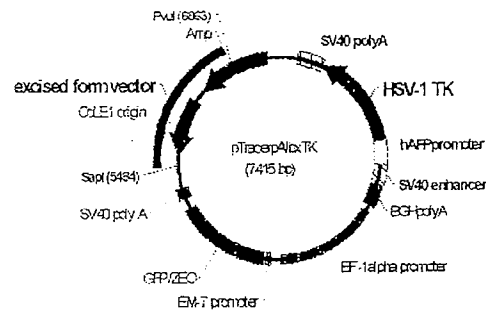


Figure 3. Non-essential areas for transgene function were excised.

Using the same approach, pGT60xTK/Fcy was also cut with appropriate restriction enzymes (Fig 4).

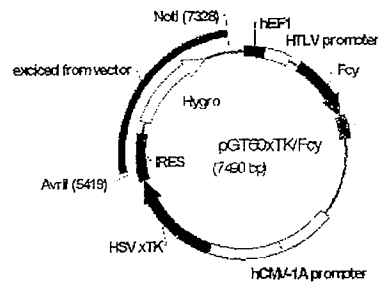


Figure 4. Non-essential areas for transgene function were excised.

Twenty micrograms of highly purified, linearized DNA was sent to the UNMC transgenic core facility for transgenic mouse production.

Sixty eight pups were born from pTracerpAlbTK. And 92 were born from the mice injected with pGT60xTK/Fcy transgene. These pups can potentially have the aforementioned plasmid DNA integrated into their genome. In order to screen these animals, their tails were clipped and DNA was extracted. A genomic PCR has been established using primers that span the promoter area and the beginning of the suicide gene. Using this PCR approach we have tested 160 DNA samples from the mouse-tails. Fig 5. shows a typical PCR gel. Lane number 8 shows a positive amplicon of 230 bp. M= molecular weight marker



Figure 5. Agarose gel stained with ethidium bromide and exposed to UV light.

We have obtained ten founder mice with these two constructs, see table 2. We are in the process of breeding these founders with male or female FVB normal mice to generate the first generation of transgenic mice.

	Number of Pups	Number of positives	% positives	Males	Females
pTracerpAlbx TK	68	5	7.35	3	2
PGT60Fcy	92	5	5.43	2	3

Table 2. Characteristics of transgenic mice.

Summary of Project Changes:

There were no new developments in this quarter.

Problems and Opportunities:

There were no new problems or opportunities this quarter.

Business Issues:

There were no new developments this quarter.

Upcoming Meetings:

No meetings were scheduled.

Technical Milestones:

No changes.

Technical Progress and Impact (November, December 2000):

1. Development of transgene constructs. The original proposal involved four constructs. Two constructs used thymidine kinase as the suicide gene. Two constructs used cytosine deaminase. One of each of these used the albumin promoter for specific expression in the liver. One of each used a CMV promoter, providing universal expression. Specific depletion of the hepatocytes would then be accomplished with immunoliposomes.

A critical review and analysis of the proposed genes and promoters led to refinement of the proposed constructs. Some of the issues are summarized here.

Thymidine kinase and male sterility? At the time of the proposal one group had observed male sterility with transgenic mice (Cohen et. al.). Since then we have heard of another group experiencing the same complication in a new set of transgenic mice using tk with an albumin promoter. This could be a fatal problem with our system. We intend to cross homozygous tk boars with wild type sows. Prodrug given to the sow would then deplete hepatocytes in the fetuses, but not the sow. Also, the herd can be expanded much more rapidly using semen from a homozygous boar. To resolve this problem, we have obtained the truncated form of tk, which is not associated with male sterility.

Albumin or alpha fetoprotein promoter for liver specific expression? For optimal utility, the suicide gene should be expressed in the liver during both fetal development and post-natal development. The initial depletion takes place at about 45 days gestation. It could be advantageous, however, to repeat the depletion of pig hepatocytes after the pig is born, or even after the hybrid liver is transplanted. In reviewing the ontogeny of albumin, however, albumin was found to be only minimally produced in the fetal pig. The alpha fetoprotein promoter was considered. Alpha fetoprotein, however, is expressed primarily in fetal development and not post-natal. AFP is also expressed in the yolk sac. This proved to not be a serious issue since the yolk sac is resorbed before 45 days gestation. The proposed constructs with albumin promoter will include AFP enhancers (but not the AFP promoter). The construct with an AFP promoter will have the silencer depleted, thought to be responsible for reduced expression post-natal. If the two constructs still lead to expression in only the adult or fetal pig, the transgenic pigs could be crossbred to contain both transgenes.

Bacteria or yeast cytosine deaminase? Most studies use the bacterial cd. There was concern, however, that the dose of 5-FU necessary to deplete the cells would also sterilize the colon of the sow, leading to problems in raising the pigs. The yeast cd, however, was found to be more than 100 times as sensitive as the bacterial cd. The gene has been ordered.

Toxicity of green fluorescent protein? The original proposed constructs would contain gfp as a reporter gene, easily detected with ultraviolet light. In addition to assisting with the development of transgenic pigs, the expression would provide an easy assay for determining chimerism within the hybrid liver. With the universal expression (CMV

promoter), the gfp could also assist with monitoring pig lymphocyte chimerism. The literature, however, has conflicting reports of possible toxicity of gfp. Though anecdotal, the development of transgenic gfp monkeys by Dr. A. Chan brought home the point. Two transgenic monkeys that produced gfp were stillborn. The only transgenic monkey to survive had the gene for gfp, as detected by PCR, but did not express the gfp. Consideration was made to have the gfp under the reverse tetracycline inducer. Expression would then be induced with an administration of tetracycline. The idea was abandoned, however, because of potential effects on the other genes and because of licensing and economic issues. We have therefore decided to exclude gfp from our constructs at this time. Dr. R. Prather has a herd of gfp pigs, which are being bred. If indicated, our transgenic pigs with the suicide genes could be bred with the gfp pigs.

Promoter for universal expression of transgene? Although the CMV promoter is generally used for universal expression of a transgene, there is increasing evidence that expression is not universal or uniform. We are currently exploring the ubiquitin promoter as an alternative.

Additional utility of transgenes in spillover technologies? Though our primary goal is to develop hybrid livers for xenotransplantation, that is considered a long-term goal. The system would be valuable, however, for spillover technologies which could provide near-term revenues. The growth of human hepatocytes or other human cells in pigs would be most useful for toxicology studies, animal models of human diseases, cost-effective production of new drugs, and the development of new vaccines.

To take advantages of these possibilities, two features have been included in the constructs. The first is the use of a universal promoter, providing expression in all cells. Specificity would be provided by immunoliposomes with tissue specific antibodies. This has been discussed previously. The second feature concerns *in vitro* purification of human cells taken from the hybrid pig. The same suicide genes used to create space for engraftment in the pig could be used to eliminate pig cells from a suspension of cells. To be most effective, however, it would be useful to have two suicide genes expressed, allowing for a double hit with prodrugs. The constructs with the universal promoter will also have two suicide genes (tk and cd) separated by an IRES gene. The two liver specific constructs will have either cd or tk. The best transgenic pigs from the two herds could then be crossbred.

The current design for the four constructs are as follows:

- a) Alb promoter-AFP enhancers-delta thymidine kinase
- b) AFP promoter (minus silencer)-yeast cytosine deaminase
- c) CMV promoter-delta thymidine kinase-IRES-yeast cytosine deaminase
- d) Ubiquitin promoter- delta thymidine kinase-IRES-yeast cytosine deaminase

The genes and sources are listed in the table:

Sequence	Source(s)
Albumin plus AFP enhancers	Currently being sequenced at Ximerex, Inc.
AFP minus first and third enhancer and silencing region	Currently being sequenced at Ximerex, Inc.
Truncated Thymidine Kinase	D. Klatzmann
Yeast Cytosine Deaminase (FCY1)	Invivogen
EMCV IRES	Clontech
CMV Promoter	Clontech

2. Development of Transgenic Pigs. The proposed project intended to produce transgenic pigs using perivitelline space injection of high titered retroviruses, a procedure developed by Gala Design, LLC. Recently the management of Gala Design requested that a license be negotiated before starting work on the project. Ximerex agreed with that suggestion. The issues have been defined and term sheets exchanged. We are optimistic that a license suitable to all will be negotiated shortly. In the event, however, that an agreement is not achieved soon, Ximerex has identified an alternative technology for developing the transgenic pigs.

Because the negotiations are taking longer than initially anticipated, Ximerex, Inc. has assumed responsibility for producing the constructs. To assist with this work, the Company recruited Dr. Carlos E. Sosa, M.D. Dr. Sosa has approximately 10 years experience with the molecular biology of viruses and producing constructs.

We have also discussed in detail work with Dr. Randall Prather, Professor of Animal Sciences at University of Missouri, Columbia. Dr. Prather has first hand experience in producing transgenic pigs using both the Gala technology and our alternate technology. Some of the pigs will be produced at the Univ. Missouri and some will be produced at the UNMC satellite facility in Oakland, Nebraska. IACUC approval for the changes has been applied for at both institutions.

At this time, it is believed that the changes in plans will not significantly affect our timetable or overall budget.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. Due to policy changes caused by a merger of the UNMC with a local hospital, the personnel and equipment previously used for fetal pig injections are no longer available. Alternative arrangements have been made.

One weakness of our technology was the inability to stain tissues for human cells. Immunoperoxidase stains have now been developed, which are specific and can be used with frozen and fixed tissue sections. We are now staining tissues collected from previous studies for cells producing human albumin.

With these sequences, restriction enzyme sites were designed to flank the promoter sequence. Specifically, Sda I will be placed about 50 bp upstream of a known DEIII transcription factor binding site. Nco I will be placed at the end of the pig promoter UTR (untranslated region).

In a similar fashion, the porcine alpha fetoprotein promoter has been identified and expanded. The sequence is being determined.

We are currently isolating the porcine ubiquitin promoter.

The suicide genes consist of delta thymidine kinase (tk) and yeast cytosine deaminase (fcy1). The delta tk plasmid was obtained from Dr. Klatzmann¹. Unfortunately, sequence analysis failed to identify the gene. We are currently producing our own truncated gene from the wild Herpes simplex virus I (HSV-1) thymidine kinase.

The fcy1 gene has been obtained from Invivogen and the sequence confirmed.

Synthesis of the constructs.

The construct consisting of AFP and fcy1 (construct b above) has been produced using the human AFP promoter. The pDriveAfp plasmid, containing human afp and SV40 enhancer was used as the backbone. The plasmid contains a Zeocin resistance gene. The yeast cytosine deaminase gene was inserted in-frame into pDrive, creating pDrive Fcy (2540 bp). The construct is illustrated in figure 2.

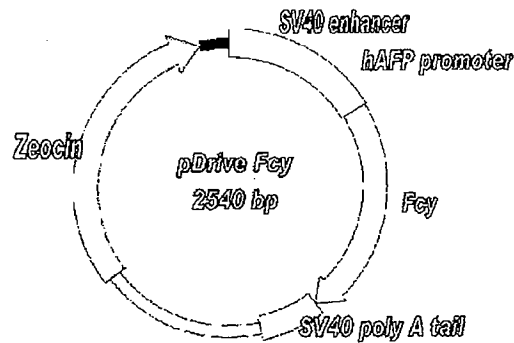


Figure 2. pDrive Fcy construct

This construct will be used for cell lines and transgenic mice. Prior to production of transgenic pigs, the human Afp promoter will be replaced with the porcine counterpart.

Using the same strategy, the HSV-1 TK gene was cloned behind the hAFP promoter, producing the pDrive HSV-1 TK (3200 bp, figure 3). The HSV-1 tk was subcloned from the commercially available pOrf HSV-1 TK (InvivoGen).

*From ATP Quarterly Report
12/01 Dec 2000 uSB
01/01-03/01*

EXHIBIT 35

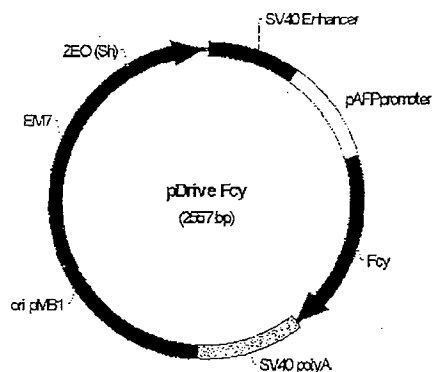


Figure 2

b. pDrive xTK

This construct contains the SV40 enhancer and the porcine albumin promoter that controls expression of a mutated version of the Herpes Simplex virus (HSV) thymidine kinase (xTK) gene (Fig 3). The gene was mutated by two rounds of site directed mutagenesis, resulting in the replacement of adenosine for cytosine at nucleotides 138 and 180. The nucleotide replacements resulted in a codon change such that a leucine replaces a methionine. These changes do not affect the enzymatic activity of the gene, since the catalytic pocket is located far away from the mutations. Expression of the TK gene in the testis would result in the infertility of the founder males, with catastrophic economical problems. The two codon changes prevent the ectopic expression of the TK gene in testis⁴.

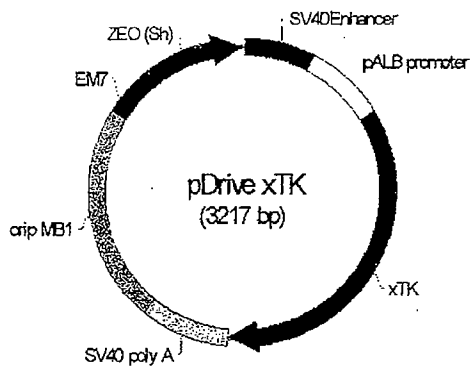


Figure 3

From ATP C. M. L. by ATP
04/01-06/01

Don H1 digestion

Aug 8-14-01

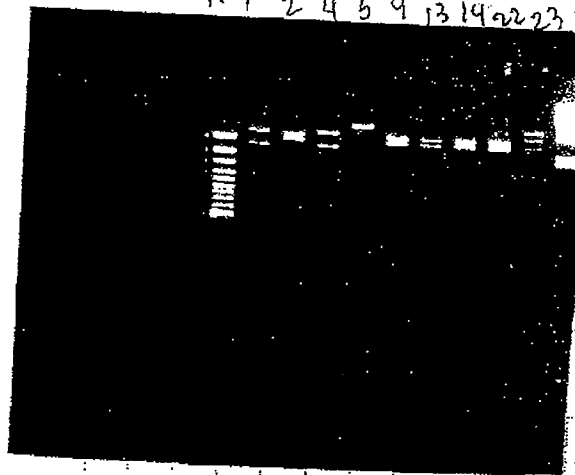
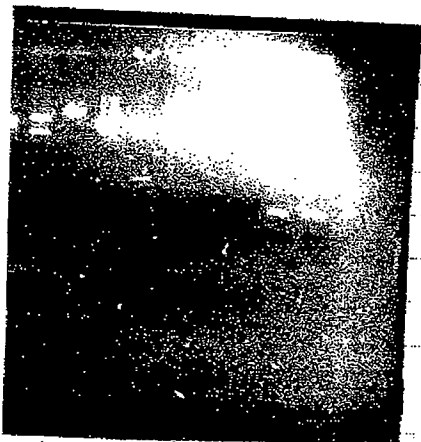


3910 bp

2853 bp

June 17-01

Digestion from H1/ECOR I



→ 3910 bp

→ 2811 bp

← 342 bp

I chose 1 and 4 for large map

You can see with transillumination that lanes 1, 2, 3, 5, 6, 7, 8 have a

Continued on Page

372 bp fragment

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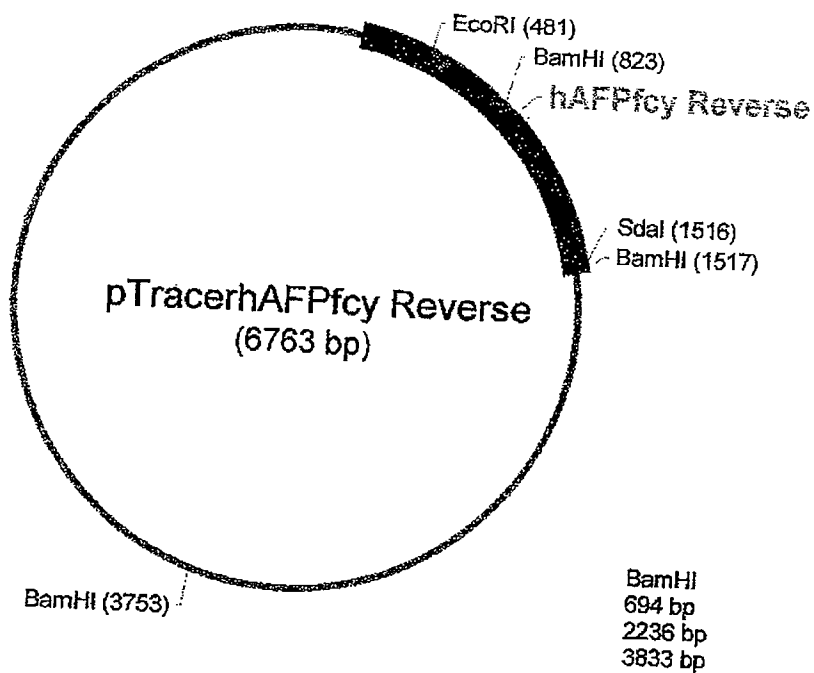
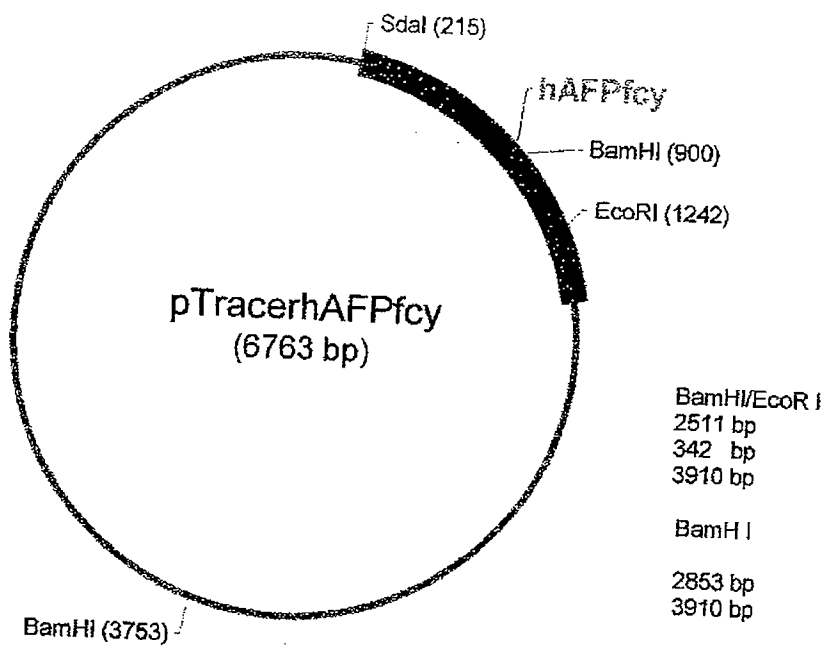
clones # 1 and 4 large map

Signed

Date

Signed

Date



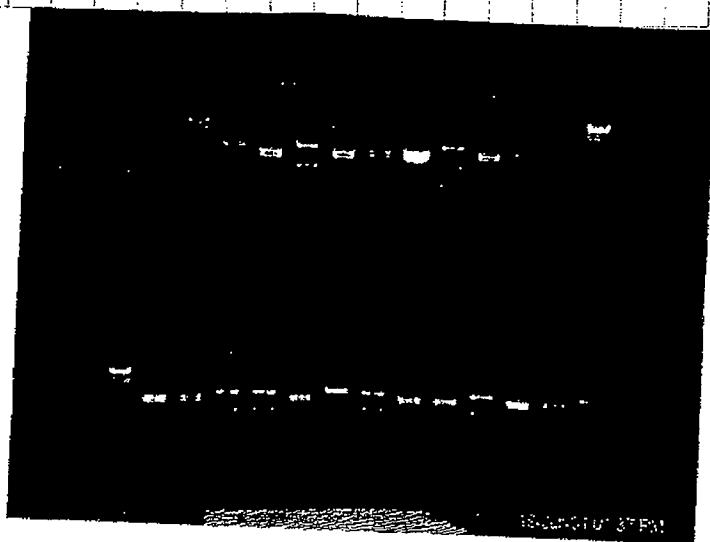
continued on Page

Screening for pTrac SK40 EN AFP ΔTK

Digested with (BamHI / EcoRI) Stock of #

1, 2, 3, 4, 5, 12, 13

is at -155



large prep with
clones #1

#2 (flipped)

I will use #1



10X Buffer 3

Eco RI

Bam HI

H₂O

DNA

1ul x 25 = 25

0.5ul x 25 = 12.5

0.5ul x 25 = 12.5

7ul x 25 = 175

2ul

Continued on Page

3

Signed _____

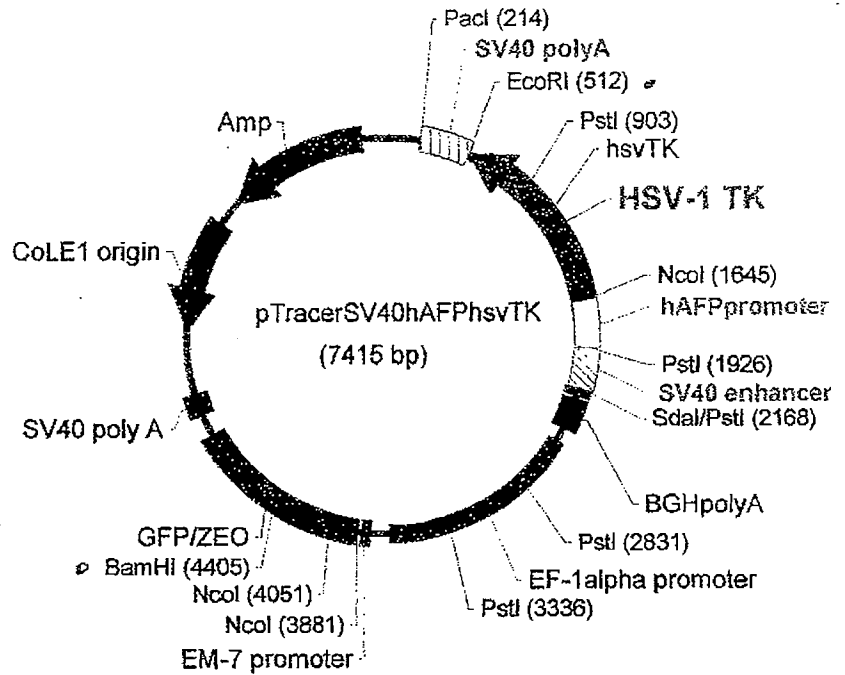
Date _____

PROJECT _____

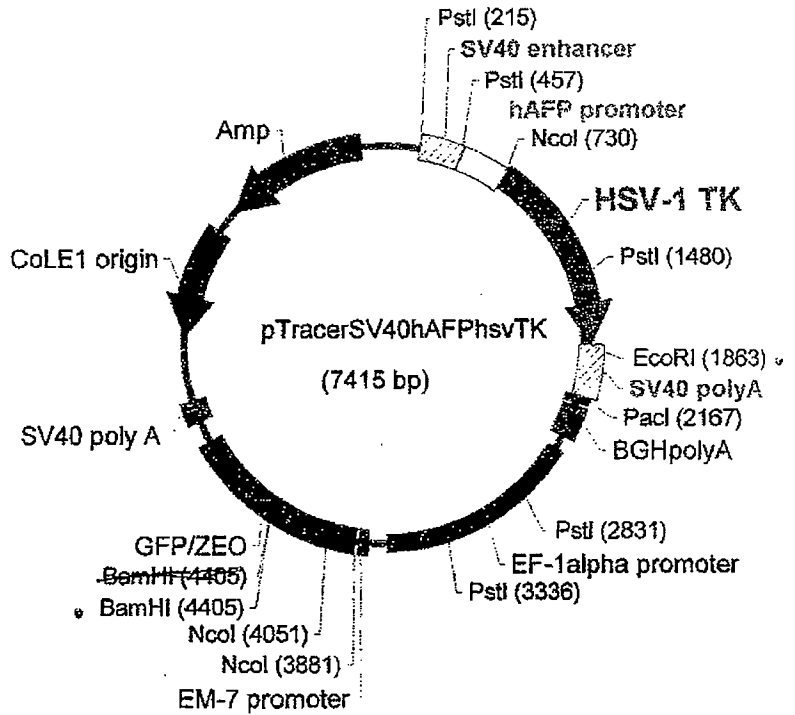
Continued From Page _____

6-28-01

Flipped
BamHI/EcoRI
3893 bp
3522 bp



Straight
BamHI/EcoRI
4873 bp
2542 bp



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Date _____

Signed _____

Date _____

6-28-01

1 = plasma surco w/AFPlus
(steige orientate)

2 = same flipped #2

3 and 4 = 5 kb when
which is number 1

MT 2 3 4 5 6



6-28-01

I started kill curve to determine which concentration we use ZEO. I put 5 different concentrations and medium alone. (500 µg, 100 µg, 200 µg, 400 µg, 1000 µg)

I plated T1B 73 and Hep G2 cells.

7-2-01
I will use 400 µg/ml of ZEO for both cell lines.

Continued on Page

Read and Understood By

5

Signed

Date

Signed

Date

3/15/01 ligating AFP A1b promoters into pGEMT vector following promega manual pg 11 of Tech manual 042 - using ~~same~~ PCR fragments from today

3/19/01 Transformation using JM109 competent cells pg 12-13 of tech manual 042 - pg 14-15 of protocol #PT3067-1 (Advantage PCR Cloning Kit User Manual)

Stock solution from Randy

IPTG - ~~250 mg in 10 ml deionized H₂O~~ make

Save at -20°

200 mg/ml (15 µl) 2g / 10 ml deionized

X gal - 20 mg/ml (50 µl) - dimethyl formamide use this much

made like this

3/19/01 using my new primers for MERT II

Continued on Page _____

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For SCThompson
3/15/01 - 5/18/01

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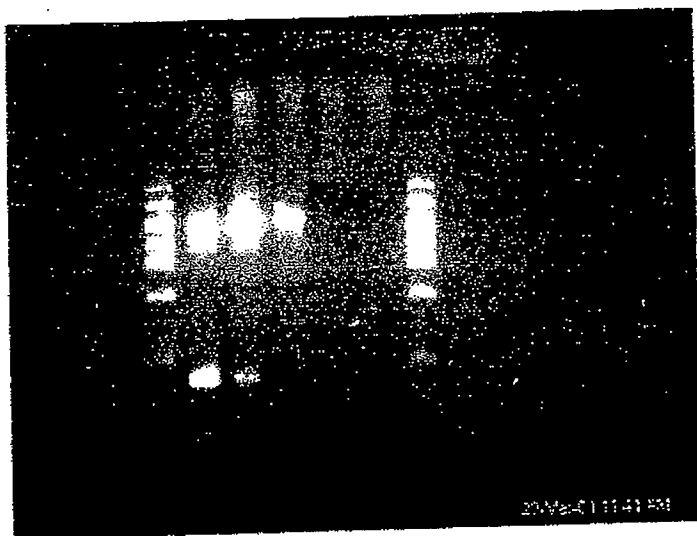
Date _____

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Date _____

3/20/01 picked white colonies out, but no
growth after 4 hrs. so leave overnight

PCR: MIMER primers



47° 51° 54° 58° 61°

3/21/01 Extracting DNA from transformed
bacteria using "Quick + Dirty mini-prep
protocol" Substituting Quagen buffers 1 + 2

Continued on Page

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7

Date

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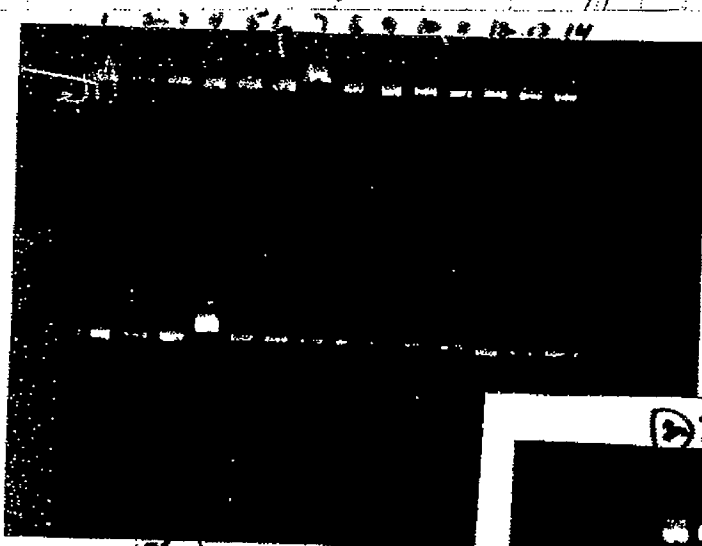
Date

3/22/01 Restriction digests after running
plasmid DNA in gel. Digested with
Not I in React 3 buffer (Gibco)

2 μ l buffer
1 μ l Not I

7 μ l ~~ddH₂O~~ e-pure H₂O

10 μ l DNA from mini prep



14 17(15)

2-300 μ l of bacteria
seeded in media
with 2 μ l ampicillin/ml



4 10 11 12 13 14

19*17

21, 27, 17

3/23/01

Extraction of plasmid DNA from
clone #s 2, 7, 9, 16, 20, 29

not enough channels for all extractions, so
only 4 extractions - Save other 2 for

sequencing with MFR II and AB AFP inter

Plasmid 2+16 in -80°C

Used Qiagen 'Plasmid Maxi Kit' protocol

pg 13-15 skip step 8 - instead use filter
to stop particulate

After elution, DNA placed at -20°C -
but protocol says 4°C

3/24/01 Pellet would not stay on tube wall -
attempted 2nd ~~centrif~~ spin w/ ethanol -

Same problem - So, placed in 50ml tube

Spin at 3400 rpm for 6 minutes.

passed calls (PK-15)

Continued on Page

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9

Signed _____

Date _____

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Date _____

3/15/01 passed cells (PK-15) - again due
to not taking enough out 3/24/01

3/26/01 Ran gel on clones - #7 may be
two plasmids + two segments? or one segment

7 4 20 29 Cuts



Continued on Page

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10

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Date

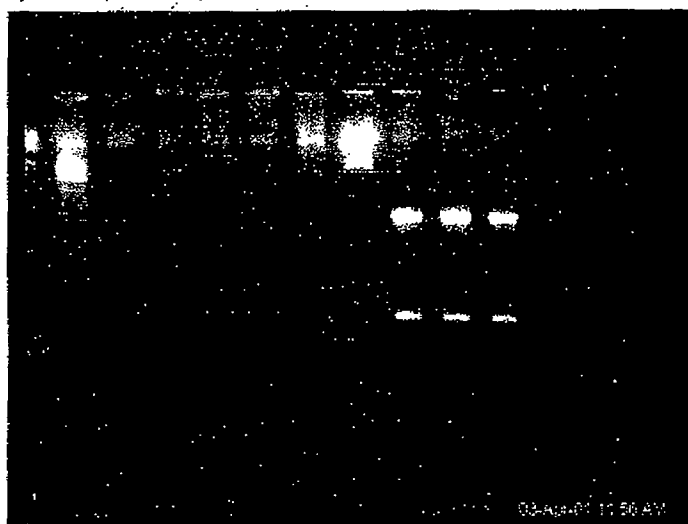
Signed

Date

4/2/01 Programs 4/4 + 4/6 run for α ABFPinter (44)
and my MER (46)

4/3/01 Addition of KCl helped dramatically
gel purification, but $< 1 \mu\text{g}$ of α ABFPinter
and $3 \mu\text{g}$ MER II.

A tailing with α ABFPinter (AA) using
Promega's A-tailing protocol pg. 8 of \times Tm042
with histone tag buffer (with MgCl)
promega's A.s., 7ul of AA



A

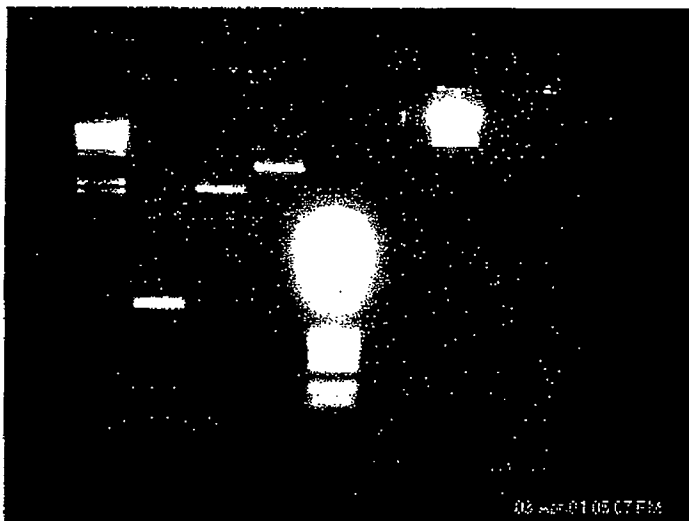
B = PKC1 + tag

Continued on Page

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ligated w/ 3 μ l of Int α , and 3 μ l MER II

4/4/01 plated bacteria w/ 100 μ l Xgal 100 μ l IPTG
after transformation. Φ (Carlos ligated + transformed)



← Int α

← MER II

4/5/01 picked colonies 1-14 (Int α) 15-26 (MER II)
seeded in ampicillin LB.

4/6/01 minipreps of 1-26 except 19 ← no 19

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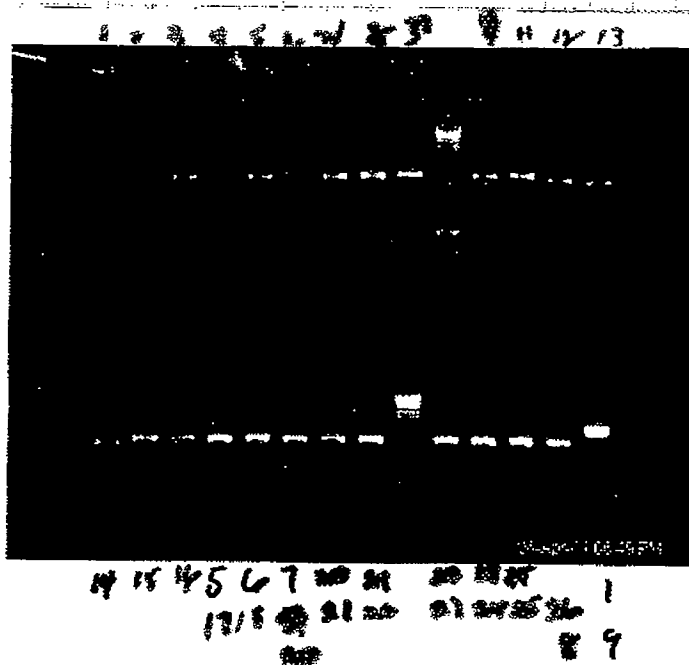
Signed _____

Date _____

Signed _____

Date _____

4/7/01 extracted plasmid DNA from the only
3 colonies of ΔTK that grew (out of 5)



~~Restriction digest of not I - yesterday~~
just plasmid DNA - yesterday

Not promising - #1 looks to have a
n/kb fragment

the digested 9 transformants plus
the 3 ΔTK to plasmids - Ran gel

Continued on Page _____

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13

Signed _____

Date _____

Signed _____

Date _____

4/16/01 Restrict digest on 8 clones 69+73 \Rightarrow

(1+2)

2ul Reat 3
1ul Not 1
12ul H_2O
5ul DNA

Run gel of digest and PCR - to see what
+ fragments size of PCR products has changed?



ran wrong
program?



1+2 digests

Media for cells: 10% FBS, 1% pen-strep
(50nt) (5ml)

Continued on Page _____

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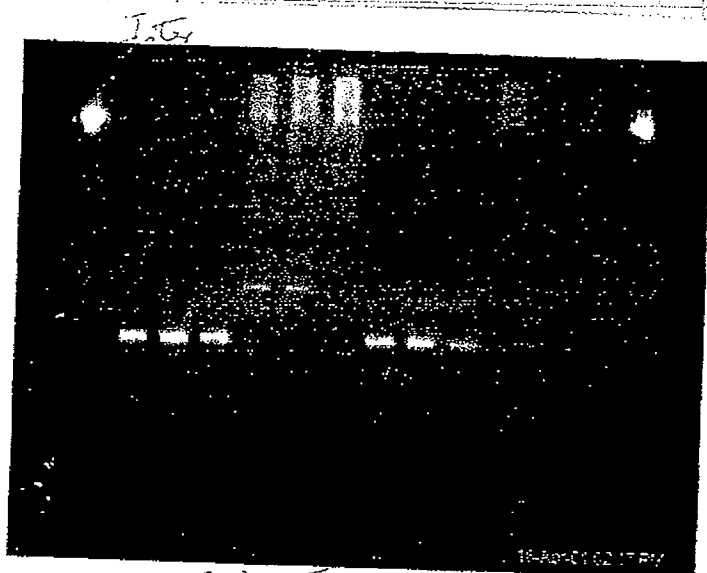
14

Signed _____

Date _____

Signed _____

Date _____



amp.
protuberance
as
substrate

granular

Continued on Page

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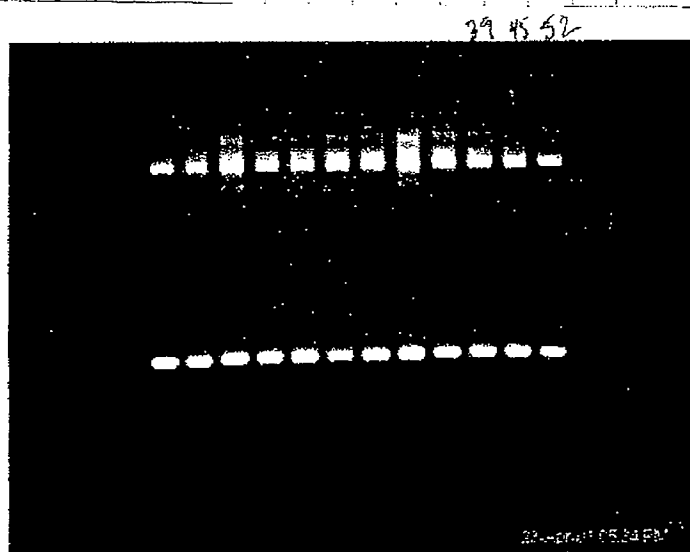
Date _____

Signed _____

4/23/01

Amplification of albumin promoter using
primers w/ SmaI, NdeI restriction sites added.

(17753, 54, 55)
(3') (3') (5')



All temps worked, all primers worked, also
old dUTPs + new dUTPs

Primer 53 had some non-specific bands

Continued on Page _____

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16

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Date _____

Signed _____

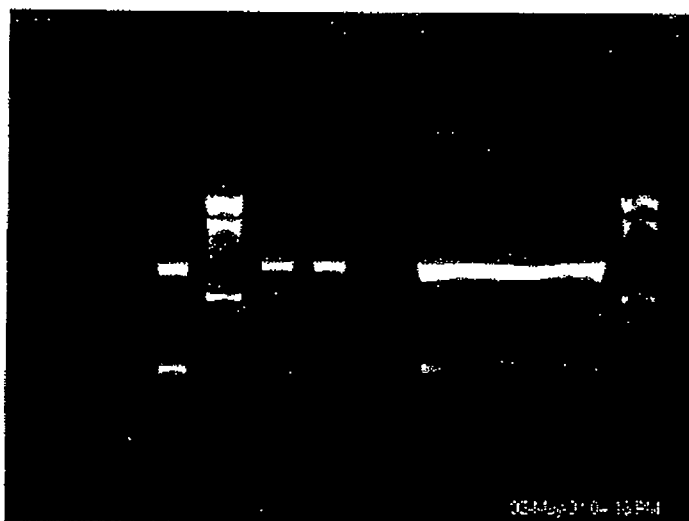
Date _____

5/1/01

Running my MER primers again - checked
on Sequencher - aligned w/ homo sequence - pretty
close - should be ~ 130 bp. Can order
new primers - did.

Left message w/ Jess @ home for him to call -
we will use their oocytes.

Talked to Nakata - Peter Wildes -
he'll look up info on room



Continued on Page _____

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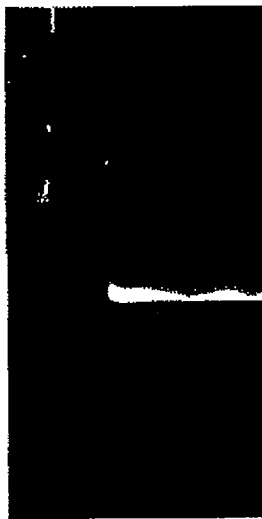
17

Signed _____

Date _____

Signed _____

5/3/01 Ran SSP + NcoI primers w/ #7, #20 OVA
for adding restriction sites + uncloned well
used. 47-bp program - all 3 temps uncloned.
Gel - purified



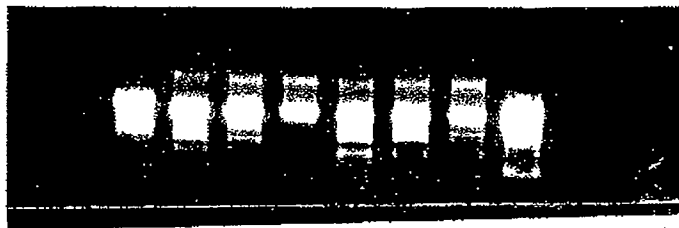
AMB



AMB ACP

NT1

5/4/01 Tried new enhancer primers MER F, F2, R



New primers

17891 842 843
MER F, F2, R

Continued on Page

Alb + AFP w/ restriction sites after restriction cutting. See opposite page

5/4/01 Amplification of enhancer region between Alb AFP using new primers and mMER primers w/ already amplified "Inter" sequence



~~PCR~~ 36° 41° 48°

P2 + R

37° 41° 48° mMER

lost picture of gel. Used program MER2

(MER 100 = .14g) (MER 200 = .26g)

5/6/01 gel purified products from 5/4/01 (mMER)

both 132 bp + 200 bp bands - ligated +

transformed using Clontech's Advantage PCR

cloning kit. On plate ~ 10pm

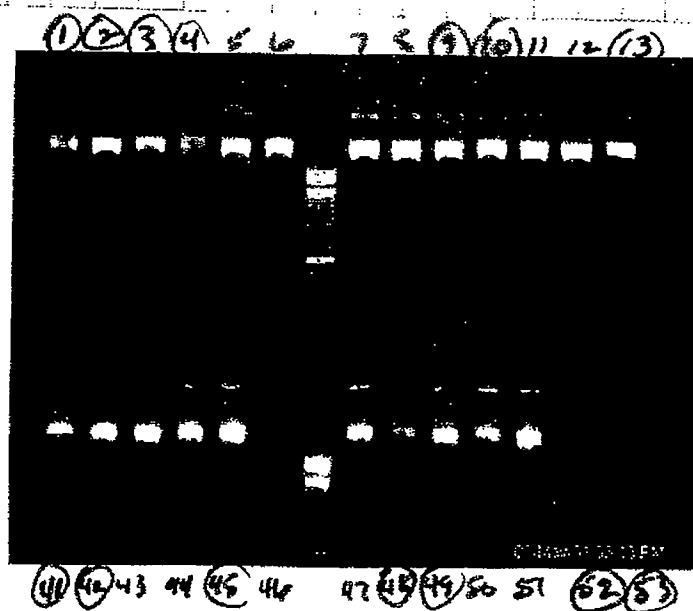
Continued on Page _____

50ul IPTG + X-gal, spread, 50ul each again + spread

Signed _____

5/7/01 colonies picked ~ 3 pm. (12 hrs later)
 after refrigerated ~ 4 hrs.
 refrigerated ~ 4 hrs at ~ 3 pm (17 hrs later)
 picked colonies ~ 8 pm. (13 f each)

5/8/01 Extracted dna by 'quick & easy' dirty
 miniprep, ran gel - all look the same
 picked 2 f each to digest w/ *ECOR I* ✓
 loading dye covers 100 bp MER (top row)



Continued on Page _____

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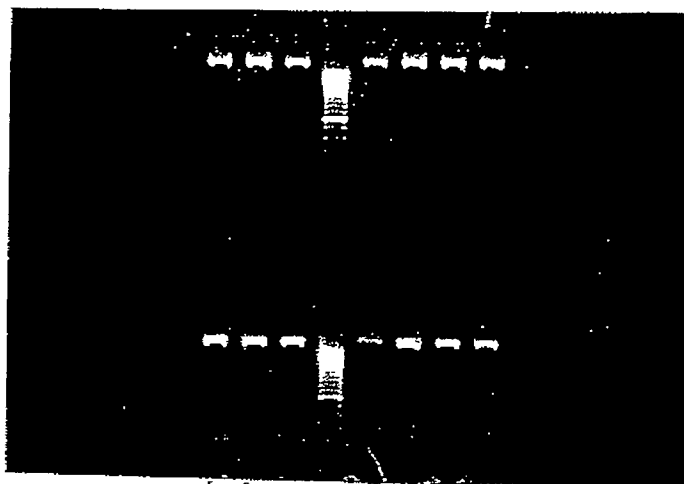
20

Signed _____

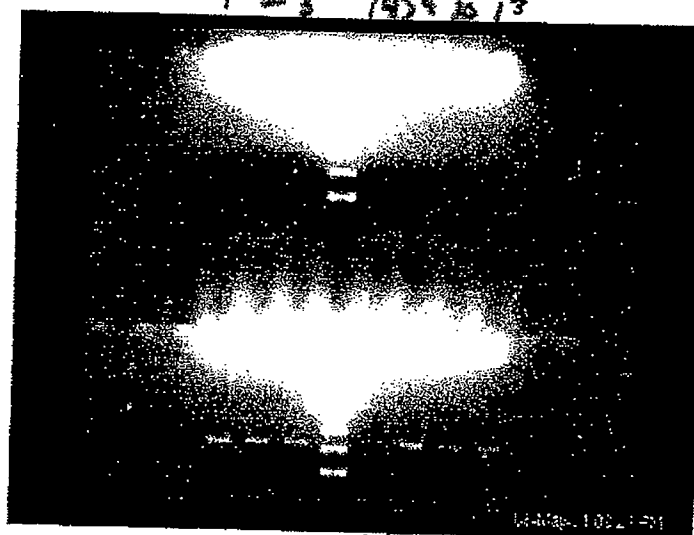
Date _____

Signed _____

Date _____



1 2 3 9 10 13



41 42 45 48 49 52 53

5/9/07 purified plasma dna w/ Promega's
Wizard miniprep kit - 30-65 ng/ul
(4, 13, 42, 49)

Continued on Page _____

Read and Understood By _____

Signed _____

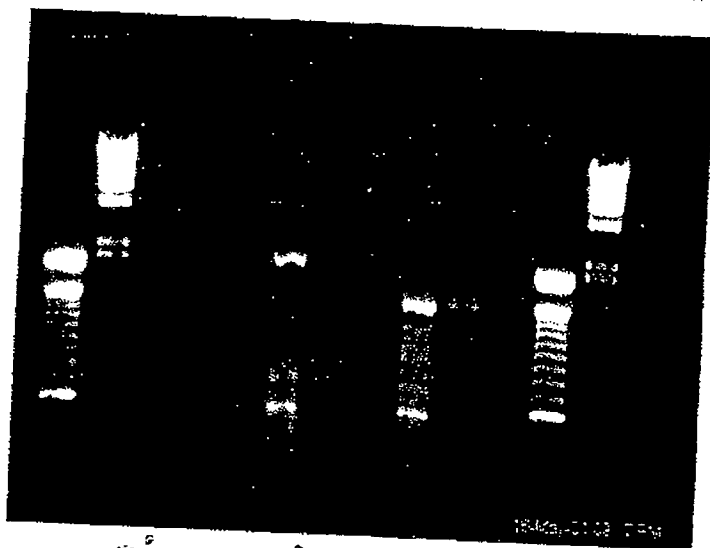
Date _____

Signed _____

Date _____

5/12/01 ordered primers (200 mer to AFP) (100 to 200 mer)
(Alb to 100 mer)

5/16/01 Ran 19358, 59 (200 to AFP) / 19360, 61 (100 to 200)
19362, 63 (Alb to 100 mer)



42°

42°

42°

58, 59

60, 61

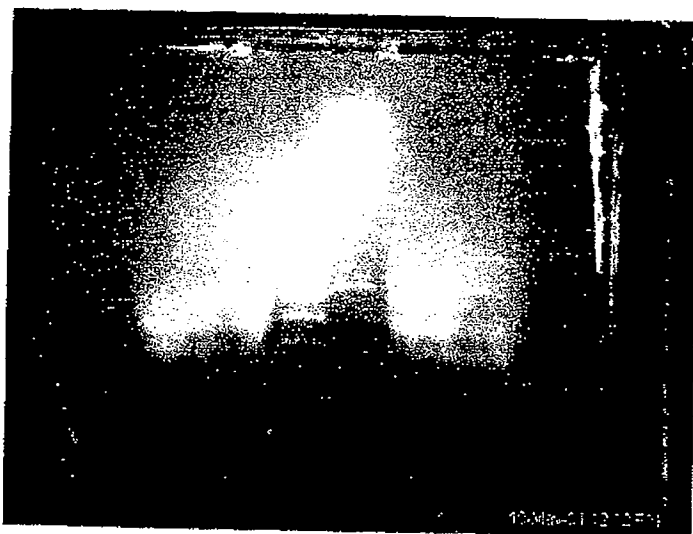
62, 63

Continued on Page _____

Read and Understood By _____

Signed _____

5/18/01 Tried amplification w/ more KCl and
on dual block



Attempted again w/ other block & same
[]s as 5/16/01



Function of liver specific promoters and thymidine kinase in stably transfected cell lines.

Mouse and human liver cell lines were transfected with constructs containing the delta thymidine kinase under the porcine albumin or alphafetoprotein (AFP) promoters. The mouse cell line (TIB73) is a line of mature hepatocytes, in which albumin is expressed. The human cell line (Huh-7) is a line of hepatocellular carcinoma cells, expressing predominantly AFP. The transfected cells were tested for thymidine kinase by Western blot (figure 4).

Western Blot Analysis of TK Expression
under the Control of Different Promoters

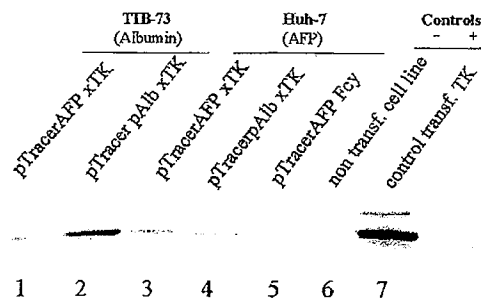


Figure 4. Western blot of transfected liver cell lines. As expected, the construct with the albumin promoter produced the greatest TK in the TIB-73 line. The construct with the AFP promoter expressed the most TK in the Huh-7 line.

To assess the function of mutated thymidine kinase, Huh-7 cells were stably transfected with pTracerpAlb xTK by selecting transfected cells using 20 ug of Zeocin. Transfected cells as well as untransfected cells were plated in duplicate in 24 well plates at a 40 percent confluence ratio. Gancyclovir was added to the cells in the culture media at different concentrations (4 uM, 8uM, and 16 uM). After 5 days, the cells were trypsinized, washed twice in PBS and stained with propidium iodine for FACS analysis.

*From ATP Quarterly Report
07/01 - 09/01*

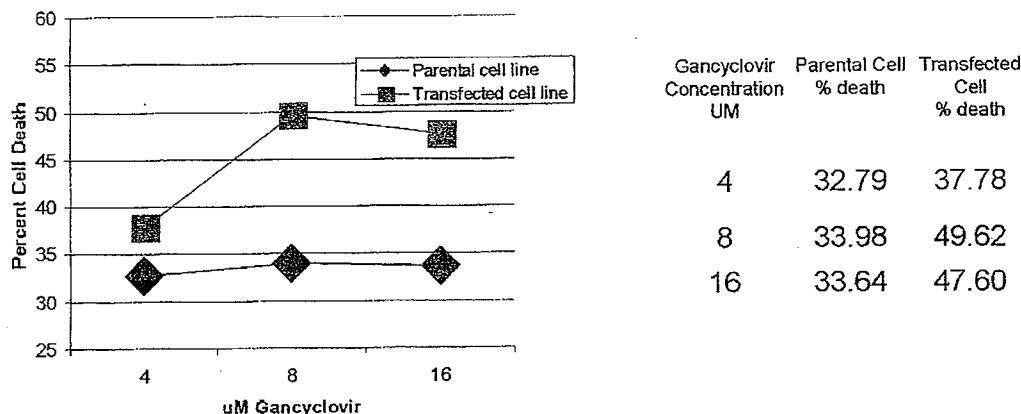


Figure # 5. Graph depicts percentage of Huh-7 cell death at 5 days incubation with different concentrations of gancyclovir in the cell culture media. The parental cell line is the un-transfected Huh-7 cell line. Transfected cells are Huh-7 cells that have been stably transfected with pTracerpAlbxTK.

The transfected Huh-7 cells were sensitive to gancyclovir between 4 and 8 uM. The TK under the albumin promoter is expressed only to a limited extent in this cell line.

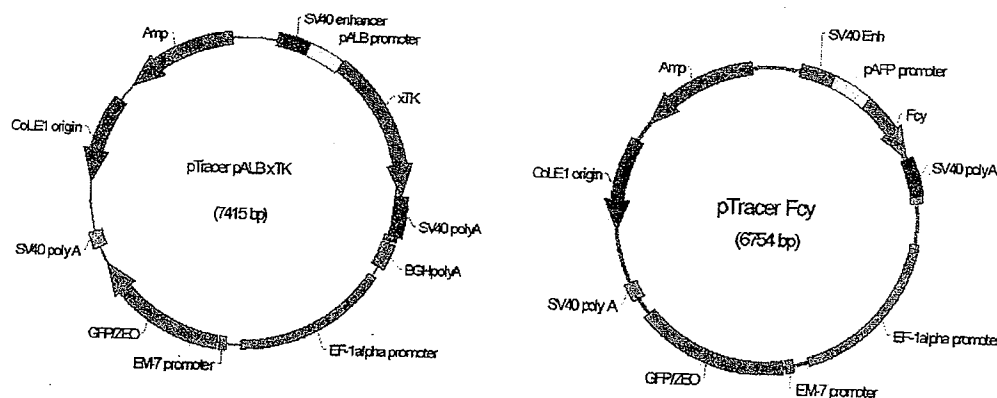
2. Development of Transgenic Pigs. As detailed in our letter of July 2, 2001, because of licensing issues, we have decided to produce transgenic pigs using nuclear transfer rather than perivitelline space injection. Arrangements have been made to produce some of the pigs at the University of Missouri, Columbia and some of the pigs in Nebraska. Fetal pig fibroblasts are transfected with the three constructs in Nebraska. The cells will be transferred into enucleated pig oocytes in Nebraska and Missouri. Significant progress has been made on both of these processes.

Fibroblasts were cultured from 35-day-old fetal pigs. After the third passage, the cells were transfected with one of the three constructs using electroporation. The cells were seeded into culture dishes and cultured for two weeks. The fluorescent colonies were isolated and tested by PCR for a portion of the construct containing the promoter and part of the suicide gene.

Fifteen stably transfected cell lines have been produced and frozen with the Albumin-delta-TK construct (Construct A, above). Each of these are PCR+.

We were in the process of isolating cell lines with the universal promoters and both suicide genes (Construct C, above), when the incubator failed. We are repeating those procedures.

Cycle 3-GFP, an improved GFP (Green Fluorescent Protein) gene fused to the Zeocin resistance gene for detection of transfected cells. It also contains the human elongation factor 1-subunit promoter (hEF-1) for mammalian expression of the Cycle 3-GFP-Zeocin fusion protein. Zeocin confers resistance allowing for stable selection of transfected cells in mammalian cell lines. The original vector contains the human CMV promoter. This promoter is of the most powerful promoters known. If not removed, it will override the activity of the tissue specific promoter that drives the suicide gene. To overcome this, we have removed the CMV promoter by using *NruI* / *EcoRV* restriction enzymes, and blunt cloned the constructs from part 1 as follows:



pTracer pALB xTK

pTracer Fcy

Using these constructs, we have transfected the HepG2 cell line, (human hepatocarcinoma cell line which expresses AFP), Huh-7 cell line (human hepatocarcinoma cell line, express AFP) and TIB73 (mouse mature hepatocyte cell line, expresses albumin). After three days in culture, transfected cells were visualized using a inverted fluorescent microscope and checked for transfection efficiency. Transfection efficiencies varied from 10-30%. Transfected cells were sent to be sorted by FACS analysis. The fluorescent positive cells were plated and Zeocin added for selection.

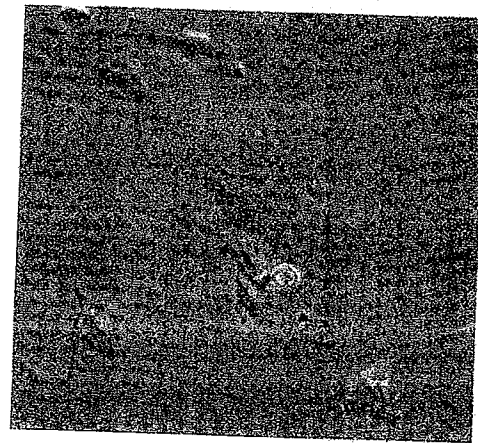


Figure 6. Confocal microscopy of Huh-7 human hepatocyte line transfected with pTracer Fcy. Two cells demonstrate fluorescence in the cytoplasm and nucleus. One cell shows fluorescence in the nucleus.

The specificity and activity of the promoters will be assessed by adding the prodrug in culture, and cell death monitored at 72 hours and 4 days after selection.

From ATP Quarterly R&D
04/01 - 06/01

We have set up this system by generating a stably transfected cell line. PK-15, a pig epithelial cell line has been transfected with pGT60 Fcy/xTK. After 2 weeks in selection media containing hygromycin B, resistant cell colonies were lifted using glass rings and trypsin, and sub-plated in 24 well plates. After the cells grew to confluence, they were plated in 6 well and then in 100 mm plates.

We set up our prodrug-killing assay by using this pGT60 Fcy/xTK PK-15 stably transfected cell lines. Six well plates containing 70 percent confluent PK15 transfected cells were added 4mM ganciclovir in the culture media. Control cells were given media alone. Cells were monitored for death. After 72 hours, cells with ganciclovir showed 60% mortality. The control cultures without ganciclovir had less than 10% mortality. At 96 hours 90% of the cells were dead. Cell mortality was measured by Trypan blue exclusion staining.

2. Development of Transgenic Pigs. As detailed in our letter to Dr. Chapekar, July 12, 2001, the best technology for producing transgenic pigs is nuclear transfer (cloning) using transfected fetal pig fibroblasts. This technology is relatively efficient, and avoids mosaicism.

3. Development of Immunoliposomes. Dr. Joshi's laboratory has produced the first liposomes. They are 100-200 nm in diameter and relatively stable. Antibodies have been ordered to produce immunoliposomes.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

Sixteen litters of pigs have been infused with human hepatocytes or bone marrow. The hepatocytes were provided by Dr. Strom, Univ. Pittsburgh. They were fresh (not frozen) and came from two human donors. The bone marrow came from 5 human volunteers. Eleven of the sixteen litters have or will go to term. Six litters have been delivered. Some of the pigs were lost shortly after delivery. The tissues and serum are being evaluated for human hepatocytes and human liver proteins. The next group of five litters will be delivered shortly.

5. Mouse Model of Hepatocyte Depletion. The above three constructs have been provided to the UNMC transgenic mouse facility. An outbreak of mouse hepatitis forced a postponement of the production of transgenic mice. It is expected to resume about August 1, 2001. This subproject is still on schedule.

Summary of Project Changes:

As described above, we intend to produce transgenic pigs using nuclear transfer technology. The nuclei in pig oocytes will be replaced with nuclei from transfected fetal fibroblasts. The oocytes are activated and implanted into the surrogate gilts.

Problems and Opportunities:

There were no significant new problems or opportunities developing this quarter.

PROJECT _____

Continued From Page _____

6-28-01

I transfected Hep G2 and TIB73 cell lines (70% confluent)

I transfected pTracer ~~ATK~~ ^{u AFP} and

pTracer u AFP fcy to both cell lines

Transfection as follows.

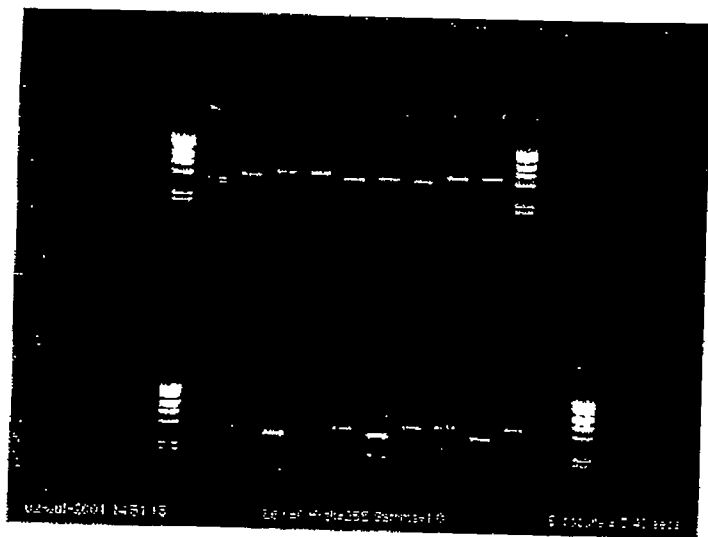
A = 7 μ l DNA + 693 μ l mediaB = 92 μ l tips + 658 μ l media } 30' incubated

After 20 hours I change to complete media

At 72 hours added 400 μ l/ml EEO

7-2-01

Screening of pTracer pATb ATK



1, 11, 13, 20

EcoRI / Bam HI

10X buffer 3 μ l \times 22 = 22 μ l
 Engine EcoRI 0.5 μ l \times 22 = 11 μ l
 Engine BamHI 0.5 μ l \times 22 = 11 μ l
 H₂O 6 μ l \times 22 = 132 μ l
 NA 2 μ l

I will freeze 1, 11, 13, 20

large scale = #20

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PROJECT _____

Continued From Page _____

	100ul	10ul	WB	TIB 73 transfection
1 = pT _{lac} AFP ΔTK				cells,
40ul DTF				48 hours after
110ul lysate				transfection were
50ul of 4x loading			204.000	harvested.
2 = pT _{lac} AFP ΔTK				2 wells per each
the same as above	112.000		122.000	construct were
55ul lysate	81.000		100.000	lysate with lys
3 = pT _{lac} Alb ΔTK				buffer and life
same as above	49.000		45.000	in tubes.
110ul lysate			→ 43.000	
4 = pT _{lac} Alb ΔTK				Efficiency of
same as above				transfection was
55ul lysate				measured by
				BFP expression
Conditions: 200 underpore transfer				≈ 10% per construct
100 V for 2 hours to run				

8-1-01

Transfections: Hep6z, TIB 73 and Huh-7 were transfected with pT_{lac} Alb ΔTK, pT_{lac} Fcy and pT_{lac} AFP ΔTK. Transfections were done using lipofectamine 6ul / 100 uls transfection mixture. Cells were put in zeocine 200 μg/ml and 400 μg/ml (for TIB 73) for selection. Cell was monitored by fluorescence. High fluorescent all died off and do not make colonies. Colonies appeared after 2 weeks. They were taken using glass rings and put in 24 well plates and then in flasks.

Continued on Page _____

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Date _____

WESTERN BLOT

8-3-01

LOW RANGE 1 2 3 4 5 6 7 8 HIGH RANGE

LOW RANGE

HIGH RANGE

204,000

122,000

102,000

50,000

1 2 3 4 5 6 7 8

2 3 4 5 6 7 8

1 = pT_{ADCM} hAEP ΔTK

2 = pT_{ADCM} pA16 ΔTK

} Transfected in TIB 73

3 = pT_{ADCM} hAEP ΔTK

4 = pT_{ADCM} pA16 ΔTK

} Transfected in Holi 7

5 = pT_{ADCM} hAEP Fcy

6 = TIB 73 with no transfection (negative control)

7 = PK15 S3

8 = PK15 S3

} stably transfected cell line with pG100

transfections were made in 6 well plates. Approximate by ^{in parallel two wells per construct}

Continued on Page

Read and Understood By

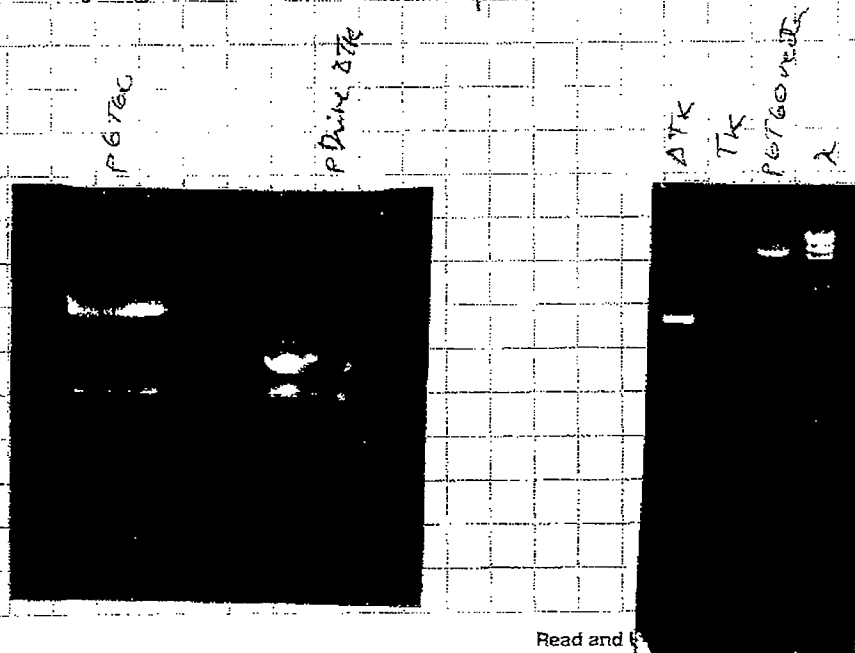
PROJECT _____

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800 ng of DNA was transfected into lipofectamine using 60-70% confluent cells (Huh7 or TIB 73). After 48 hours cells were checked for fluorescence transfection and 20-30% of efficiency was found. Cells were harvested at 48 hours using lysis buffer. Two wells (of 6 well plate) were lysed using 1 ml of lysis buffer per well after cells were snap frozen with liquid nitrogen.

Subcloning of pGT60

8-8-01

Changing the TK for an Δ TK

Continued on Page

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Ligation 8-12-01

	(1)	(2)
	+	-
Buffer 10x	2 ul -	2 ul -
Env [T]	1 ul	1 ul
DNA pET60	1 ul -	1 ul -
DNA ATK	3 ul -	-
H ₂ O	13 ul -	16 ul -
TOTAL	20 ul	20 ul

8-10-01

ELECTROPORATION

TIB 73 and F2 3rd pass fibroblasts were transfected with pTwin-Fcy, AFP and Alb ATK.

1 Flask of fibroblasts trypsin, wash 1x PBS. Then diluted in 2 ml of PBS. Fibo 100% confluent. The same for TIB 73 24 hours from passage.

Take 0.9 ml and put in cuvettes, 20 ul of each construct was added.

Continued on Page

Read and Understood By

6

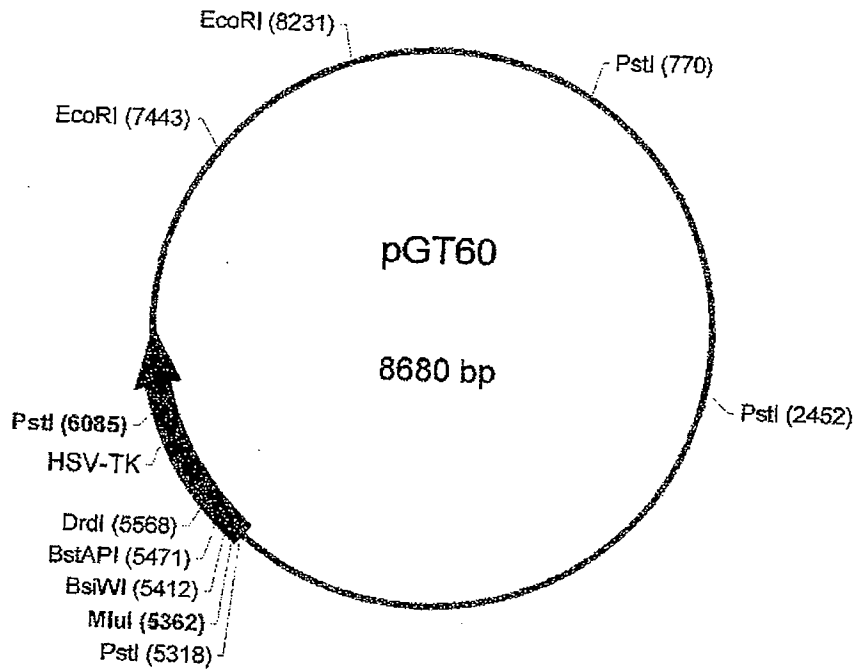
Signed

Date

Signed

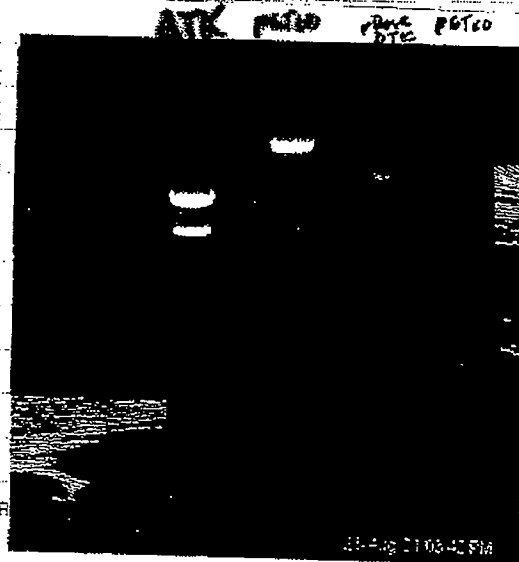
Date

Cloning pGT60 / ΔTK



MLO / SNAI restriction

Buffer	5 ul	Buffer	5 ul
MLO		MLO	
SNAI	3 ul	SNAI	3 ul
DNA (pGT60)	10 ul	primase	10 ul
H ₂ O	32 ul	primase	10 ul
TOTAL	50 ul	H ₂ O	32 ul
		TOTAL	50 ul

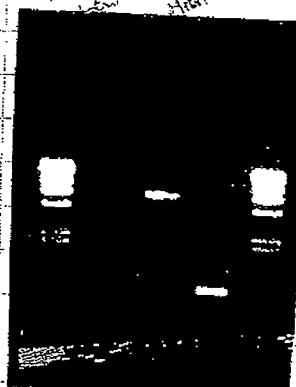


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Date _____

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Date _____

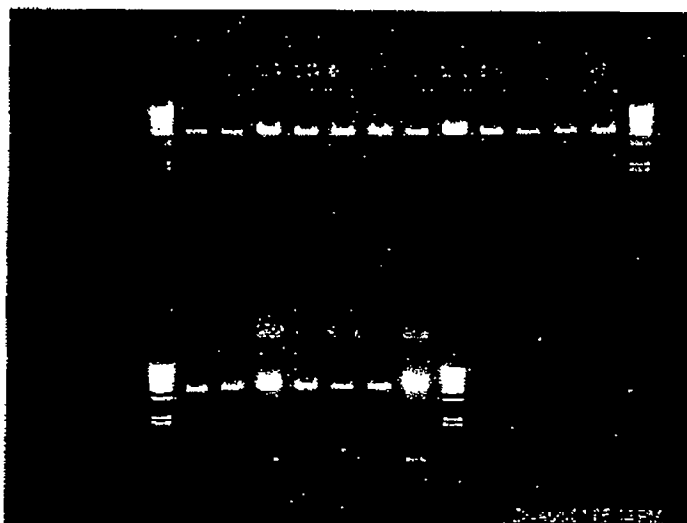


REACTION

	①	②	③	④
10x buffer	1 ul	1 ul	1 ul	1 ul
lipase	1 ul	1 ul	1 ul	1 ul
pG60 high	1 ul	1 ul	1 ul	1 ul
pG60 low	—	—	5 ul	—
DTK	3 ul	5 ul	—	—
H ₂ O	4 ul	2 ul	1 ul	7 ul
TOTAL	10	10	10	10

EcoR_x Rx x 22

EcoR _x 10x Buffer	1 ul x 22 = 22 ul
EcoR _x	0.5 ul x 22 = 11 ul
H ₂ O	6 ul x 22 = 132 ul
DNA	2.5 ul
<u>TOTAL</u>	<u>10 ul</u>



→ 7892 bp

→ 788 bp

1, 8 and 15

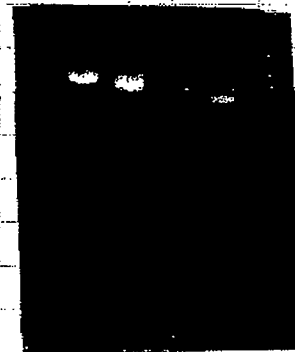
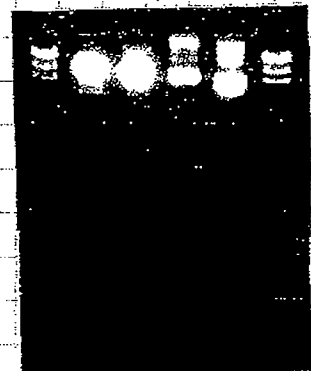
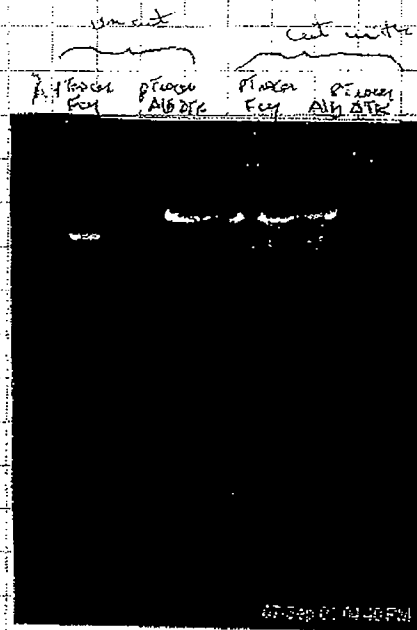
check in freezer

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Signed

Linearizing pTracer Fcy and A13 ΔTK
using PstI

Sep 7 - 01



pTracer Fcy = 6754

pTracer A13 ΔTK = 7415

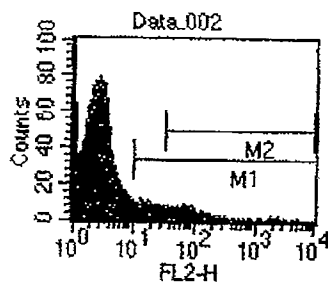
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Sept 4, 01

Huh-7 transfected early with pTet-ALOTR

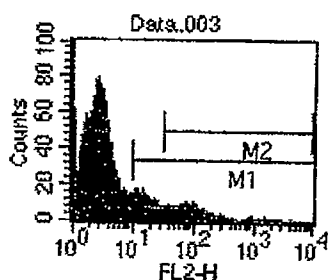
Histogram Statistics

4 days of
passaging

File: Data.002
 Sample ID: 09042001ty
 Tube:
 Acquisition Date: 04-Sep-01
 Gated Events: 10000
 X Parameter: FL2-H (Log)

Log Data Units: Linear Values
 Patient ID:
 Panel:
 Gate: No Gate
 Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991C	10000	100.00	100.00	13.74	3.47	800.49	2.69	1
M1	10, 991C	1282	12.82	12.82	88.17	37.41	336.50	32.78	12
M2	34, 991C	622	6.22	6.22	161.58	81.97	255.96	68.85	66

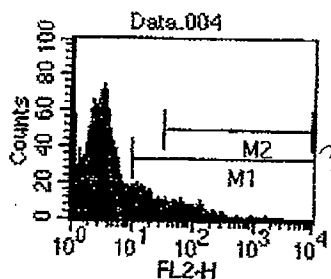


File: Data.003
 Sample ID: 09042001ty
 Tube:
 Acquisition Date: 04-Sep-01
 Gated Events: 10000
 X Parameter: FL2-H (Log)

Histogram Statistics

Log Data Units: Linear Values
 Patient ID:
 Panel:
 Gate: No Gate
 Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991C	10000	100.00	100.00	15.65	4.04	587.34	2.92	1
M1	10, 991C	1708	17.08	17.08	77.28	36.06	274.19	29.69	10
M2	34, 991C	792	7.92	7.92	146.20	87.92	202.87	74.99	74



File: Data.004
 Sample ID: 09042001ty
 Tube:
 Acquisition Date: 04-Sep-01
 Gated Events: 10000
 X Parameter: FL2-H (Log)

Histogram Statistics

Log Data Units: Linear Values
 Patient ID:
 Panel:
 Gate: No Gate
 Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991C	10000	100.00	100.00	18.72	4.80	543.98	3.40	1
M1	10, 991C	2084	20.84	20.84	77.19	33.41	276.15	24.80	11
M2	34, 991C	841	8.41	8.41	165.06	92.62	191.26	76.35	35

I have to repeat experiment because the controls overgrow.

Continued on Page _____

Read and Understood By _____

10

Signed _____

Date _____

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~~9-7-01~~ - ~~Prep~~ Prepare cells for transfection:
I used ~~about~~ F2 cells of 3rd passage.

Cells were split into 6 well plates. After 24 hrs will be transfected with Gene Janner.

9-8-01

Gene Janner transfection:

300 μ l of gene janner
10 μ l of DNA
500 μ l of DMEM (plain)

overnight transfection, change media 9-8-01
9-10-01 Cells do not seem to make GFP maybe 0.5% gl
Lot F₁ 8-13-01 were thawed (9-9-01)

9-10-01 cells were electroporated.

80% confluent flocks (2) were trypsinised in
800 μ l of cells 2 bottles 0.300 & 0.50,
in ice PBS.

Cells are put into 100 mm plate with w/gh
9-11-01

Few cells glancing. Few cells alive.

changing media (without ATB)

9-10-01 Two vials of lot: F2 8-06-01
were thawed.

9-11-01 60% of cell confluency - change
media. (Lot F2 8-06-01)

9-13-01

Throw away gene janner transfection from ~~now~~
or I will use only electroporation Read and Understood By

8-13-01 Cells of F2 8-06-01 do not look good. Thawed every

Signed

Date

Signed

Date

9-12-01 One vial of fibroblasts 2nd pass ^{F2} (no lot)
(cells look very good and trypsinized)

2 cuvettes prepared 400 μ l
Electroporation

9-13-01 no lot electroporation looks ~~ok~~, lots
of cells survive.

I will add 400 mg/ml ZEOcin today.

9-12-01 lot F1 8-13-01 transfection I added
400 mg/ml ZEO (40 μ l in each 10ml plate)
drop by drop

9-13-01
~~According~~ According to experiment of different
dilutions of ZEOcin = 400 mg/ml seems
to be the minimal dose to kill pf fibroblasts
after 6 days

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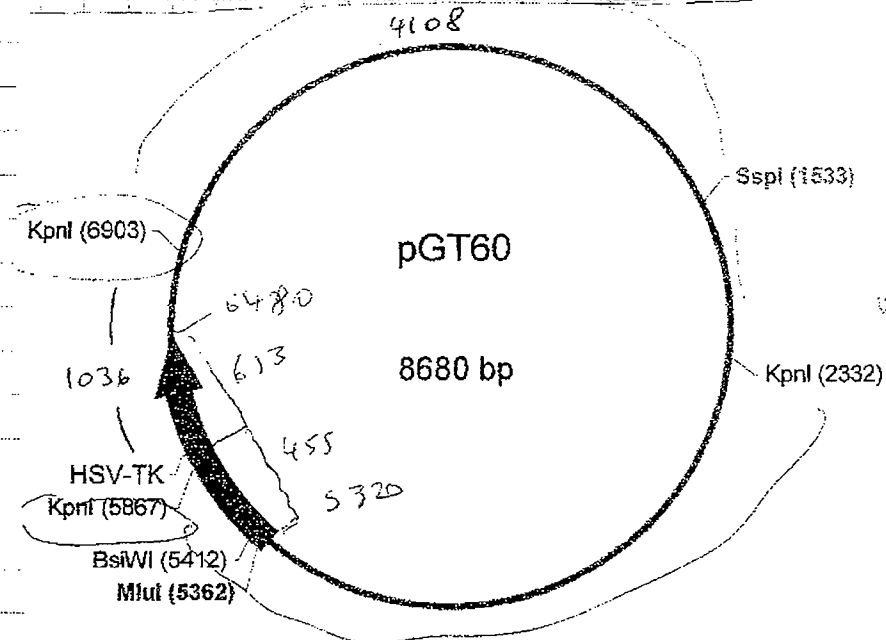
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Signed _____

Date _____

Signed _____

Date _____



3535

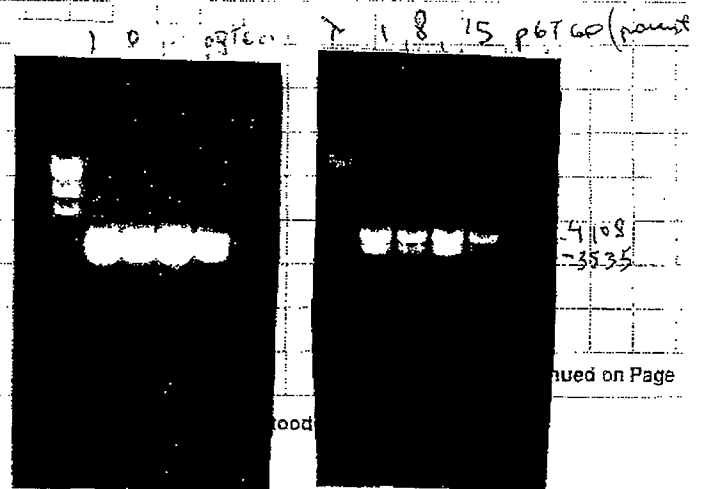
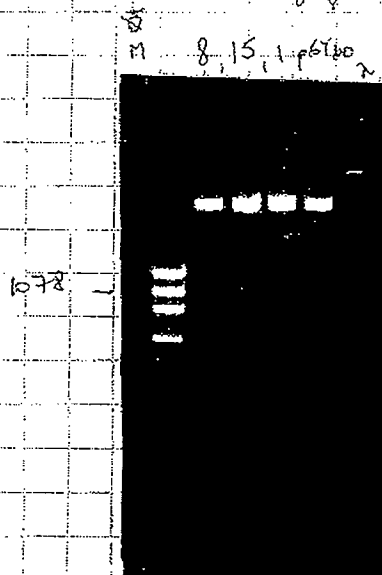
3535

4108

1036

Digestion with KpnI to confirm orientation
of ΔTK (4108 - 3535)

Sent for sequencing
using TKFLIA-F
clone # 1, 8, 15



Continued on Page

Signed

Date

Signed

Date

Gancyclovir with
cell line

Notebook No. _____

Continued From Page _____

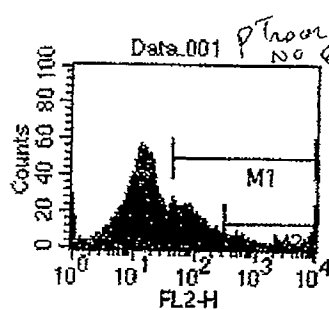
245

5 days

HIV-7

9-17-01

Histogram Statistics



File: Data.001

Sample ID: 009172001cs

Tube:

Acquisition Date: 17-Sep-01

Gated Events: 10000

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

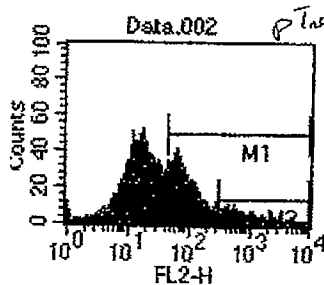
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Gate: No Gate

Total Events: 10000

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M1	45, 991C	2893	28.93	28.93	555.61	149.11	289.88	100.90	69
M2	319, 991C	518	5.18	5.18	2611.75	1264.23	116.97	736.53	9910

Histogram Statistics



File: Data.002

Sample ID: 009172001cs

Tube:

Acquisition Date: 17-Sep-01

Gated Events: 10000

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

Patient ID:

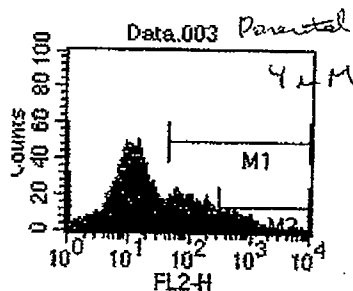
Panel:

Gate: No Gate

Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991C	10000	100.00	100.00	115.99	34.08	366.20	27.38	12
M1	45, 991C	3778	37.78	37.78	277.04	129.32	238.28	90.58	69
M2	319, 991C	675	6.75	6.75	1090.36	773.61	116.71	632.09	425

Histogram Statistics



File: Data.003

Sample ID: 009172001cs

Tube:

Acquisition Date: 17-Sep-01

Gated Events: 10000

X Parameter: FL2-H (Log)

Log Data Units: Linear Values

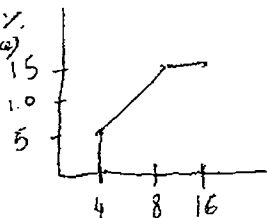
Patient ID:

Panel:

Gate: No Gate

Total Events: 10000

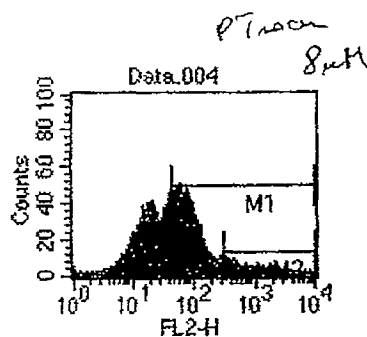
Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991C	10000	100.00	100.00	148.18	26.96	433.71	17.23	1
M1	45, 991C	3279	32.79	32.79	423.82	178.75	252.66	135.77	60
M2	319, 991C	866	8.66	8.66	1265.74	795.82	144.97	649.38	9910

Decl %
(difference)gancyclovir
concentration
μM

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Signed

Date

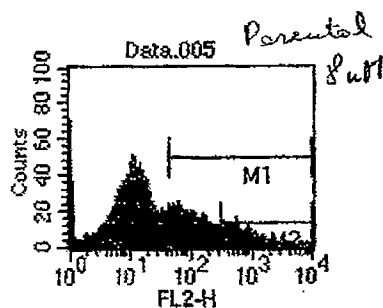


File: Data.004
Sample ID: 009172001cs
Tube:
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Histogram Statistics

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991C	10000	100.00	100.00	129.54	45.20	374.98	44.51	41
M1	45, 991C	4962	49.62	49.62	239.10	113.05	281.05	85.05	51
M2	319, 991C	623	6.23	6.23	1234.97	803.62	126.62	626.43	331

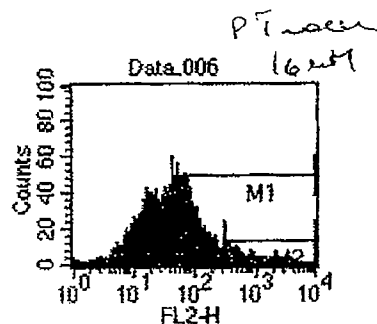


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Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Histogram Statistics

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991C	10000	100.00	100.00	135.41	27.84	394.37	18.35	41
M1	45, 991C	3398	33.98	33.98	371.33	174.94	233.98	135.77	51
M2	319, 991C	909	9.09	9.09	1055.47	715.53	139.60	572.55	331

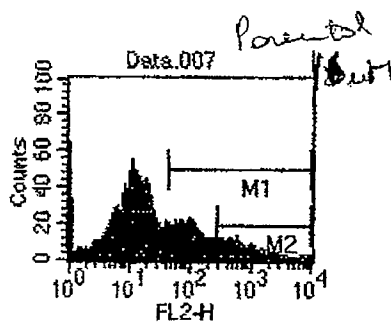


File: Data.006
Sample ID: 009172001cs
Tube:
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

Histogram Statistics

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991C	10000	100.00	100.00	113.33	42.00	362.43	42.55	41
M1	45, 991C	4760	47.60	47.60	214.98	109.65	269.08	82.79	51
M2	319, 991C	559	5.59	5.59	1108.00	745.23	125.24	562.34	331



File: Data.007
Sample ID: 009172001cs
Tube:
Acquisition Date: 17-Sep-01
Gated Events: 10000
X Parameter: FL2-H (Log)

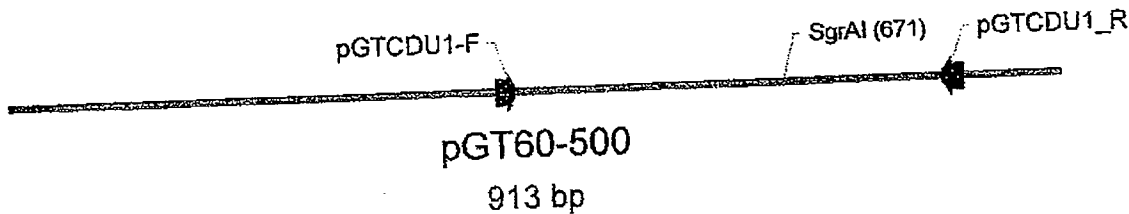
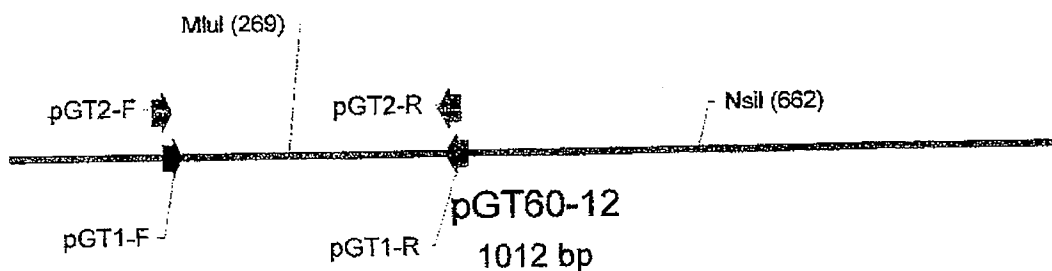
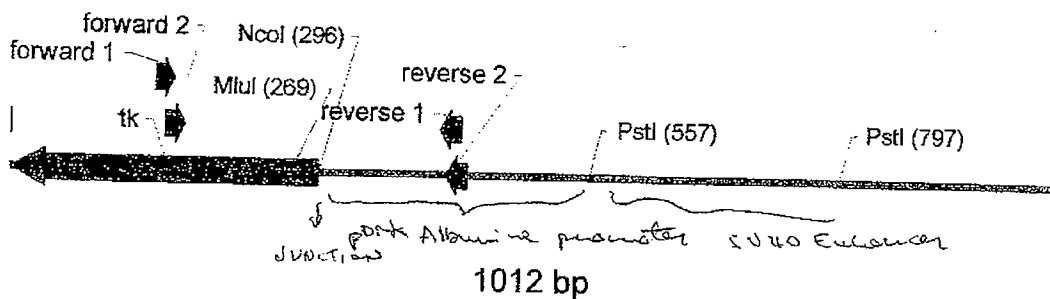
Histogram Statistics

Log Data Units: Linear Values
Patient ID:
Panel:
Gate: No Gate
Total Events: 10000

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak C
All	1, 991C	10000	100.00	100.00	133.55	28.02	370.22	18.43	41
M1	45, 991C	3364	33.64	33.64	369.16	176.35	217.21	134.56	51
M2	289, 991C	982	9.82	9.82	986.37	682.79	130.49	559.82	331

9-25-01

PCR to screen for pTher Alb ΔTK
in cell lines on Thompson mice



Signed

Date

Signed

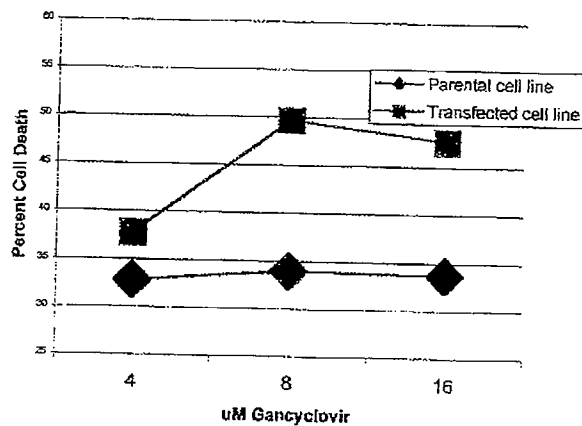
Date

PROJECT _____

Continued From Page _____

Assessment of the Porcine albumin promoter and mutated Thimidine Kinase (xTK) activity in a cell culture system:

Huh-7 cells were stably transfected with pTracerpAlbxTK by selecting transfected cells using 20 ug of Zeocin. Transfected cells as well as untransfected cells were plated in duplicate in 24 well plates at a 40 percent confluence ratio. Gancyclovir was added to the cells in the culture media at different concentrations (4 uM, 8uM, and 16 uM). After 5 days, the cells were trypsinized, washed twice in PBS and stained with propidium iodine for FACS analysis.



Gancyclovir Concentration uM	Parental Cell % death	Transfected Cell % death
4	32.79	37.78
8	33.98	49.62
16	33.64	47.60

Figure# 1. Graph depicts percentage of Huh-7 cell death at 5 days incubation with different concentrations of gancyclovir in the cell culture media. The parental cell line is the un-transfected Huh-7 cells. Transfected cells are Huh-7 cells that have been stably transfected with pTracerpAlbxTK.

This experiment shows a moderate difference in gancyclovir sensitivity between transfected cells (carrying pTracerpAlbxTK) and the parental cells (un-transfected). The Huh-7 cell line is an immature hepatocarcinoma cell line which expresses high titers of AFP. It was predicted that the expression of the albumin promoter in this cell line would not be dramatic; due to the lack of maturation of this cell line they do not express high titers of albumin. However, we were able to see differences in the percentage of cell death between transfected cells, 47% of the cells were dead, and un-transfected cells, 34% of the cells were dead. This suggests a moderate activity of the xTK in stably transfected cells. This is important because it allows us to monitor the activity of the genetically engineered xTK and conclude that the two point mutations performed in the gene have not rendered the TK enzyme inactive.

age

Signed _____

Date _____

Signed _____

Date _____

EXHIBIT 43

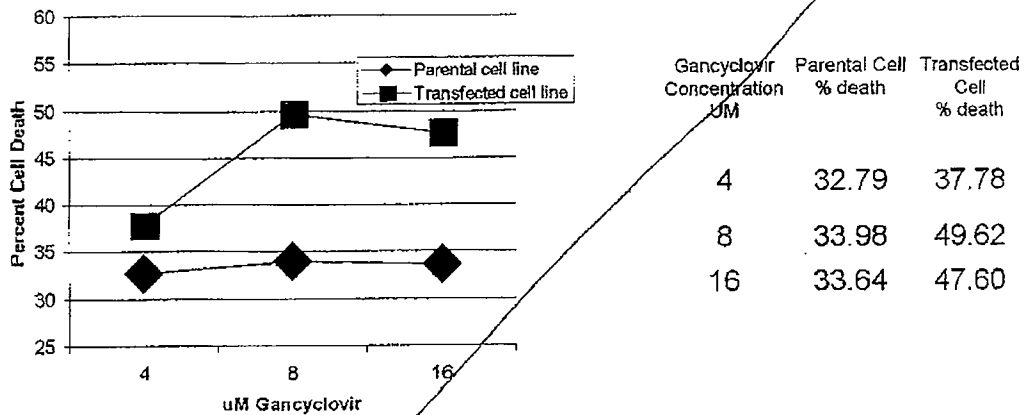


Figure # 5. Graph depicts percentage of Huh-7 cell death at 5 days incubation with different concentrations of gancyclovir in the cell culture media. The parental cell line is the un-transfected Huh-7 cell line. Transfected cells are Huh-7 cells that have been stably transfected with pTracerpAlbxTK.

The transfected Huh-7 cells were sensitive to gancyclovir between 4 and 8 uM. The TK under the albumin promoter is expressed only to a limited extent in this cell line.

2. Development of Transgenic Pigs. As detailed in our letter of July 2, 2001, because of licensing issues, we have decided to produce transgenic pigs using nuclear transfer rather than perivitelline space injection. Arrangements have been made to produce some of the pigs at the University of Missouri, Columbia and some of the pigs in Nebraska. Fetal pig fibroblasts are transfected with the three constructs in Nebraska. The cells will be transferred into enucleated pig oocytes in Nebraska and Missouri. Significant progress has been made on both of these processes.

Fibroblasts were cultured from 35-day-old fetal pigs. After the third passage, the cells were transfected with one of the three constructs using electroporation. The cells were seeded into culture dishes and cultured for two weeks. The fluorescent colonies were isolated and tested by PCR for a portion of the construct containing the promoter and part of the suicide gene.

Fifteen stably transfected cell lines have been produced and frozen with the Albumin-delta-TK construct (Construct A, above). Each of these are PCR+.

We were in the process of isolating cell lines with the universal promoters and both suicide genes (Construct C, above), when the incubator failed. We are repeating those procedures.

*From ATP Quarterly Report
7/01 - 9/01*

Technical Progress and Impact (October through December 2001):

1. Development of transgene constructs.

There were no changes or developments regarding the constructs.

Development of stably transfected cell lines.

A. Production of Fetal Pig Fibroblasts (FPF)

Two 35-day-old fetal pigs were obtained by Caesarian section from one adult sow. The three centimeters long fetuses were minced into 1-mm³ pieces and 0.5% trypsin was added, and after half hour incubation the isolated cells were filtered. After two washes in PBS the cells were seeded in 170 cm² flasks with DMEM plus 10% fetal calf serum. After one week, when the cultures were confluent, cultures were split into 3 flasks. This is counted as a passage 1. This sub-culture procedure was repeated twice. Cells of passage 3 were frozen at -150°C.

B. Electroporation:

Between 8 to 10 million FPF cells were transfected by electroporation using the following method:

1. FPF were diluted in 0.4 ml of PBS and poured into a 0.4 cm electroporation cuvette and placed on ice for 10 minutes.
2. 10-20 ug of linearized pTracerpALBxTK plasmid was added to the cell mixture
3. The electroporator was set at 0.300 mV and 0.950 pF, and the cells were shocked for 31 usec.
4. Following electroporation, cells were seeded immediately onto a 10 mm plastic dish containing DMEM with 10% FCS.
5. After 48 hours of incubation the media was changed to a DMEM containing 400 ug/ml of Zeocin.
6. After three weeks in culture, colonies were scored for fluorescence intensities and isolated using glass rings. Colonies isolated this way were transferred to a 24-well-plate.
7. After another 4 days of incubation the cells were trypsinized and divided in two fractions. One fraction was frozen in DMSO and the other was put back in the well for further amplification of the culture followed by DNA extraction and PCR screening for presence of the transgene.

C. Screening for the transgene.

In order to confirm that the cells that we have selected with GFP/Zeo selection system also have the suicide gene stably integrated in the cell genome, we performed PCR on DNA isolated from transfected FPF. We designed primers using a junction area spanning the promoter and the beginning of the suicide gene as template. This *sis* one area is highly unlikely to have homology to any other chromosome regions in the genome and produce false positives. PCR conditions were optimized using a gradient capable PCR thermocycler. The primers and the area chosen for amplification are depicted in Fig. 1.

From ATP Quarterly Report
10/01-12/01

2. Development of Transgenic Pigs. The license with the University of Missouri using nuclear transfer technology developed by Dr. Randall Prather has been finalized and signed by both parties. Transfected fibroblast lines were sent to Dr. Prather. They have begun the nuclear transfer. In culture, the nuclear transfer units (embryos) looked healthy. They were placed in surrogate sows. At this time, however, it is too early to report their outcome.

Scott Thompson, in Omaha Nebraska, has optimized conditions for nuclear transfer in our laboratory. We are now implanting 40 to 60 embryos per sow and implanting 1 or 2 sows per week. Through the end of March, three sows were implanted with embryos. One sow was bred, the other two were not bred. A follow-up ultrasound examination (done in April 2002) showed all three sows to be pregnant. While the significance of the bred sow will need to wait until the pigs are examined by PCR, the pregnancies in the unbred sows are presumably due to the implanted embryos. This observation is very encouraging.

3. Development of Immunoliposomes. There are no new technical developments. Our original proposal involved immunoliposomes containing antibodies to the asialoglycoprotein receptor. That would provide specificity for liver cells. However, we were unable to find available antibodies. There were also patent issues involving the polyethylene glycol linkage of antibodies to the liposomes. As an alternative, therefore, we will produce liposomes containing asialoglycoprotein. The ligand containing liposomes should bind to the liver cells containing the corresponding receptor¹. There do not appear to be any intellectual property issues with this approach. According to Dr. Joshi, these will likely be more stable than immunoliposomes.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug.

There are no new developments.

5. Mouse Model of Hepatocyte Depletion.

We have continued breeding the F1 and F2 generations of transgenic mice and we also continue with the screening process by PCR. Table 1 summarizes the stage of the research on these animals.

	pGT60ΔTAK		pTracer pAlbΔTK	
Founder	5		3	
	Tested positive/total	Untested	Tested positive/total	Untested
First Generation (F1)	16/94	30	8/20	22
Second Generation (F2)				21

From ATPQ weekly
Report for 1/02-3/02

5. Mouse Model of Hepatocyte Depletion.

At this time we have 13 transgenic mice in the F2 generation that have been tested by PCR. The bands are either moderate or intense. We are testing the six mice with intense bands to establish if they are homozygous. Three of these mice are males. In the experimental plan, homozygous males are to be bred with wild type females, producing heterozygous transgenic fetal mice in a normal maternal host.

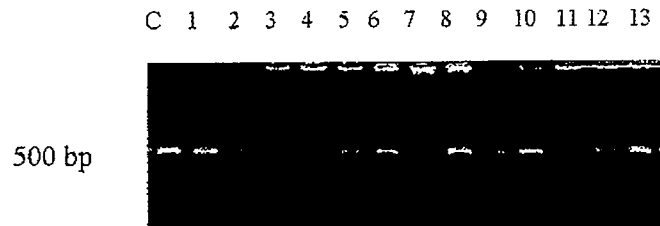


Figure 3. PCR gel stained with ethidium bromide and photographed under UV light. Different intensities in 500 base pair band suggests presence of homozygous DNA in the sample. Sample C: Positive Control. Sample 1, 5, 6, 8, 10, 13 are possible homozygous mice.

We are testing F1 mice by Western blots and with gancyclovir to confirm expression of the thymidine kinase and function of the protein.

Projects 6, 7, 8. No progress at this time.

*From ATP Quarterly Report
to 4/02 - 6/02*

Corynebacterium

7/25/0

Transgenic mice = ~~the~~ Alb-Tu

Control - rare necrotic cell

EXHIBIT 47

291/292 ALT 212 vesicular A in cytopl.

293/4 ALT 88 centrilobular nuclear condensation

6 - ALT 27 centrilobular cells large nuclei
weight 1045 vesic. A in cytoplasm

3

1

Continued on Page

Read and Understood By

William E. B. 7/25/02

Pregnant mice given 0, 25, 50 or 100 μ g/kg Concylovir
Day 14(?)

484-1 Lung + Heart

484-2 Liver - 4+ cyt. vacuolization - EMH

484-3 Liver - 4+ cyt. vac. all necrosis - EMH ok, P. & artefact

484-4 Liver 4+ cyt. vac.

484-5 Liver 4+ cyt. vac.

~~484-6~~ Liver 4+ cyt. vac. - EMH

484-7 Liver 4+ cyt. vac. - EMH

484-8 Liver 4+ cyt. vac. - EMH

486-1 Liver 3+ cyt. vac.

486-2 Liver 4+ cyt. vac.

#2 Liver 3-4+ cyt. vac.

486-3 Liver 3+ cyt. vac.

486-4 Liver 3-4+ cyt. vac.

486-5 Liver 3+ cyt. vac. - EMH

486-6 Liver 3+ cyt. vac. - EMH

A Liver autolyzed, infected

B Liver 2+ cyt. vac. - EMH - results good

C Liver - 4+ cyt. vac.

Continued on Page

Read and Understood By

William E. Beckler 8/12/02

Signed

Date

Signed

Date

D Liver 2+ cyt vac.

E Liver Perimeter 2+ cyt vac. looked good, most was autolyzed.

F Liver Perimeter good 2+ cyt vac. Central 30% necrotic.

G Liver. Most looked good 2+ cyt vac.
Patchy areas necrotic or autolyzed.

H Liver. Large areas autolyzed.
Some areas = pyknotic nuclei + focal lipid.

I Liver - Generalized - pyknotic nuclei
2+ cyt vac. Incr. EMA or lymph.

J - Liver - autolyzed.

K Liver - Periph = pyknotic nuclei
Central - Autolyzed.

L Liver - Periph. pyknotic nuclei + 2+ cyt vac.
Most is autolyzed.
Normal skeletal muscle.

Continued on Page

Read and Understood By

William E. Berchman

8/12/02

Signed

Date

Signed

Date

Technical Progress and Impact (January through March 2002):

1. Development of transgene constructs.

Development of stably transfected cell lines.

The previous progress report described problems encountered with the construct containing both thymidine kinase and cytosine deaminase under a universal promoter. Two new constructs were produced, containing only one suicide gene under a different universal promoter, CMV. The plasmids are pTracerCMV2xTK and pTracerCMV2Fcy.

The pig fibroblast lines were transfected with the new plasmids and have been expanded in culture. We anticipate using these lines for nuclear transfer in the next quarter.

2. Development of Transgenic Pigs.

Last quarter, one sow was implanted with embryos in Missouri and three were implanted in Nebraska. The Missouri sow was initially pregnant, but the fetal pigs resorbed at 4 weeks. Two of the Nebraska sows showed evidence of pregnancy by ultrasound, though the pigs appeared to be lost at 4 and 6 weeks.

These sows were autopsied. One sow (#752) showed 6 implantation sites. The other sow (#88) had 5 implantation sites. A normal fetal pig was recovered from that sow.

The transgene of this fetus was pTracer pAlbxTK, with the mutated thymidine kinase under an albumin promoter. The vector also contained the green fluorescent protein under the control of a ubiquitous promoter. Below (figure 1) is the pig photographed under ultraviolet light. Note the presence of green fluorescence in the area of the liver and in the hooves.

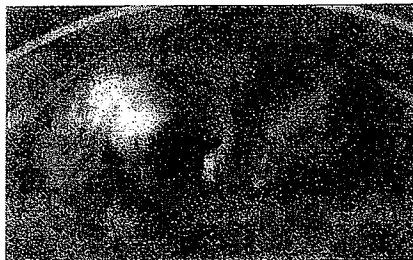


Figure 1. Six week old fetal pig from sow #88 under ultraviolet light.

Western blot analysis was performed using an antibody directed against herpesvirus thymidine kinase (TK). Different samples were processed by mechanical disruption using a buffer containing detergent and proteinase inhibitors. Figure 1 shows a positive band of approximately 44 kilodaltons, corresponding to the molecular size of the TK protein. A liver sample from fetal pig #88 was subjected of this treatment (lane 1) as well as lung tissue (lane 2) from the same animal.

From ATP Quarterly Report
Period 4/02 - 6/02
(Heading is incorrect. Report made
on 7/27/02).

Ovoalbumin
44 KD

Figure 2. Western Blot analysis. M: molecular weight marker; lane 1: lysate of liver from fetus #88; lysate of lungs of fetus #88. Thymidine kinase is shown with a molecular weight of 43 Kd

These observations are significant to this project for two reasons. First, this pig demonstrates that the thymidine kinase is expressed in the liver as predicted. Second, at 6 weeks, this pig is the approximate age of fetal pigs that will be treated with prodrug (gancyclovir). This pig demonstrates that the thymidine kinase is expressed at this stage of development.

During the second quarter, Dr. Prather at the Univ. Missouri transplanted 483 embryos into 4 sows (all with mutated thymidine kinase under the albumin promoter). One has recycled. One sow is too early to evaluate. Two of the sows are still pregnant and are scheduled to deliver on July 26, 2002 and August 15, 2002.

During this quarter, Scott Thompson has transplanted 651 embryos into 10 sows (all with the mutated thymidine kinase under albumin promoter construct). Three sows are too early to evaluate. Four sows have are resorbing fetal pigs. Three sows are still pregnant and are due on August 10, 16, and 17.

3. Development of Immunoliposomes. There are no new technical developments.

4. Infusion of Normal Fetal Pigs with Human Hepatocytes, Immunoliposomes, Prodrug. There are no new developments.

EXHIBIT 49

Technical Progress and Impact (July through September 2002):

1. Development of transgene constructs.

Development of stably transfected cell lines.

We mentioned in the last report the transfection of pig cells using the new transgene constructs that contain a suicide gene under the control of a powerful ubiquitous promoter (CMV). Pig fibroblasts were transfected with the new constructs (pTracerCMVxTK and pTracerCMVFcγ). We are in the process of isolating stably transfected colonies. We have encountered a problem with the transfection of the construct (pTracerCMVxTK). This construct has shown very low colony production efficiency. Four transfection experiments have been performed but very few colonies have been produced at this time. This may be due to a deleterious effect of the herpesvirus thymidine kinase. It may be expressed in large quantities and thus it would be toxic. We consider that the CMV promoter is one of the most powerful promoters known and the expression of the enzyme is very high. However, we continue trying and are transfecting a higher number of cells to circumvent this obstacle.

2. Development of Transgenic Pigs.

Last quarter, several sows were implanted with embryos in Missouri and two of them were pregnant. Dr. Prather used stably transfected cell lines that we produced in our lab in Nebraska. The cells have the pig albumin promoter which is liver specific and controls the expression of the mutated thymidine kinase. These two sows have delivered; they have given birth to two healthy transgenic piglets from one sow and one transgenic piglet from the other. The piglets born in both experiments are males, which is very advantageous for the development of the project. They are very healthy and continue to thrive. Samples of ear and umbilical cord were used for DNA extraction and PCR was performed to confirm the presence of the transgene. The three piglets were shown to be positive for the transgene. Fig 1 shows the characteristic band of the promoter thymidine kinase spanning 400 bp.

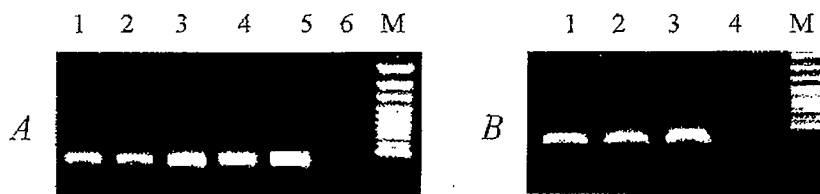


Figure 1. PCR gel stained with ethidium bromide and photographed under UV light.

The 400 base pair band shows presence of the transgene. A- First born piglets: lane 1 and 2 is Xim1, lane 3 and 4 is Xim 2, lane 5 and 6 are positive and negative controls respectively. B- second born piglet: lane 1 and 2 are Xim3, lane 3 and 4 are positive and negative controls respectively. M: molecular weight marker.

From ATP Quarterly Rpt
7/02 - 9/02

We have performed 600 embryo transfers in our lab; these embryos have been implanted into 9 sows, on weekly bases. Three types of constructs were used: pTracerAFP_{Fcy}, pTraceCMV_{Fcy} and pTracerCMV_{xTK}. Of the nine sows implanted, three sows have shown a pregnancy by ultrasound scanning with due dates of November 2, 9, and 23. Table 1 summarizes these experiments.

	Date	Transgene	Sow ID	Embryos transferred/ Parthenotes	Ultrasound	Due date
1	7/12/2002	AFP Fcy	90-1	41/7	Pregnant	11/2/2002
2	7/19/2002	AFP Fcy	424-1	46/12(5and7)	Pregnant	11/9/2002
3	7/25/2002	CMV Fcy	465	72	not pregnant	11/15/2002
4	7/26/2002	CMV Fcy	65-0	65	Possible Pregnancy	11/16/2002
5	8/2/2002	CMV Fcy	652	85	Pregnant	11/23/2002
6	8/23/2002	CMV xTK	263	50/8	not pregnant	12/14/2002
7	8/30/2002	CMV xTK	654	49	not pregnant	12/21/2002
8	9/6/2002	CMV Fcy	456-1	72/9	Possible Pregnancy	12/28/2002
9	9/13/2002	AFP Fcy	231-1	75/9	not pregnant	10/4/2003

Table 1.

3. Development of Immunoliposomes.

Our collaborator, Dr. Shantaram Joshi of the Univ. Nebraska Medical Center, has performed experiments to establish the ability of gancyclovir-loaded immunoliposomes, using a monoclonal antibody against pig MHC class I, to kill porcine cells that have been stably transfected with thymidine kinase. Figure 2 summarizes these experiments. We can see from the graph that the empty liposomes do not kill the cells. The concentration of gancyclovir that seems to be optimal to kill pig cells in this *in vitro* model is 50 μ M. Also the activity is very specific because the cells that do not contain the transgene (PK-15) are only minimally affected by the immunoliposomes containing prodrug.

8

PROJECT _____

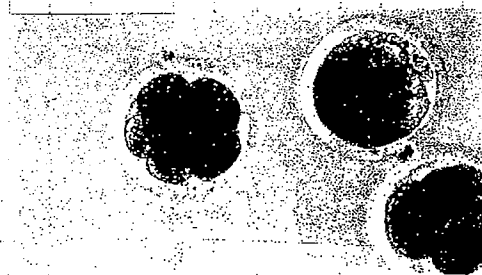
Notebook No. _____

Continued From Page _____

Bill transported 72 embryos w/ no parths
to Oakland for 10:00 transfer into sow 465

7/24/02 Carlos transported 65 embryos (miscar
yesterday) for transfer at 10:00 to sow 65-0
No parths 10 cleaned of 19 F/A Sw
6 cleaned of 46 F → A

7/27/02 One 7/24 parth at 8 LMS



7/30/02 New TL HEPES made piped at 7:43 on
new
script probe

Raised temp of CO₂ incubator to 60°C to
burn of volatile organics.

Continued on Page _____

Read and Understood By _____

Signed _____

Date _____

Record of embryo transfer (9/23/02)

Nuclear transfer by using Ximerex's cells as donors

Date	Donor cells	Sources of oocytes	# Recipient	Status of recipient	Culture time of embryos	Embryos transferred	Cycle (date)	Ultrasound check (date)	Comments
3/28	F1-12	Bomed	O035	1	1	139	NO (4/23)	Degenerating (4/23)	
4/3	F1-8	Bomed	O094	1	1	148	Yes (5/8)		Cycle on d 35
4/4	F1-3	Bomed	O156	0	1	127	No (7/22)	See big skeleton fetuses and heartbeat (7/22)	2 live male piglets (1005g, 1870g) were born on 7/29
4/24	F2-G4	Bomed	O122	1	1	124	No (7/22)	See 3 big skeletons (7/22)	One live piglet (470g) was born on 8/19
5/1	F2-D3	Bomed	O142	1	1	84	Yes (5/27)		Cycle on d 26
8/7	F1 (8-13)	Bomed	Y248	1	1	104	Yes (9/11)		Cycle on d 33
8/8	F2-G9	Bomed	5	0	1	31	Yes (8/28)		Cycle on d 20
8/14	F2-D6	Bomed	Y261	0	1	119	No (9/23)	See fetuses (9/23)	

Beth Harrison

From: Lisa Hemmendinger
Sent: Thursday, September 19, 2002 12:06 PM
To: 'beschorner@ximere.com'
Cc: Beth Harrison; Dale Hoscheit
Subject: RE: Ximere New Patent Proofread

Thanks, Bill. We'll file it today.

-----Original Message-----

From: beschorner@ximere.com [mailto:beschorner@ximere.com]
Sent: Thursday, September 19, 2002 10:16 AM
To: Lisa Hemmendinger
Subject: Ximere New Patent Proofread

Lisa,
The patent is very well done. I didn't have any major changes. Several trivial changes were made. The document is in redline format showing my changes. Regarding prodrugs that cross the placenta, I was able to identify three, including gancyclovir. They were added. The corresponding references are in a comment. Call me (Cell (402)659-6552) if there are additional issues. Otherwise go ahead and file it.
Thanks!
Bill

William E. Beschorner, M.D.
President and Chief Scientific Officer
Ximere, Inc.
2614 N. 161 Ave.
Omaha, NE 68116-2461
Tel: (402)559-2235 Fax: (402)445-2535
Website: www.ximere.com

Beth Harrison

From: Lisa Hemmendinger
Sent: Wednesday, September 18, 2002 8:01 AM
To: 'beschorner@ximrex.com'
Cc: Dale Hoscheit; Beth Harrison
Subject: draft application



provisional
application.DOC

Dear Bill:

The revision of your draft application is attached. Please review it for accuracy and completeness. I've embedded a few questions for you in the text.

We typically do not file claims with provisional applications. However, the specification does support the claims you drafted.

Remember I will be out of the office on Friday, so we should file the application on Thursday at the latest.

Lisa

Lisa M. Hemmendinger
Banner & Witcoff, Ltd.
1001 G Street, N.W.
Washington, D.C. 20001

direct phone 202-508-9291
fax 202-508-9299

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Lisa Hemmendinger

From: beschorner@ximrex.com
Sent: Monday, September 16, 2002 10:28 AM
To: Lisa Hemmendinger
Subject: RE: New Patent

Sounds Great!
Bill

----- Original message -----

>Date: Mon, 16 Sep 2002 08:29:16 -0400
>From: "Lisa Hemmendinger" <Lhemmendinger@bannerwitcoff.com>
>Subject: RE: New Patent
>To: <beschorner@ximrex.com>

>

>Dear Bill,

>

>I'm about half-way through revising it, and I'd like you to take a look at it before it's filed. I would guess either tomorrow or Wednesday; Thursday at the absolute latest, as I won't be here on Friday. Does this work for you?

>

>Lisa

>

>-----Original Message-----

>From: beschorner@ximrex.com [mailto:beschorner@ximrex.com]
>Sent: Monday, September 16, 2002 8:19 AM
>To: Lisa Hemmendinger
>Subject: New Patent

>

>

>Hi Lisa,

>I wanted to schedule a meeting with a company that is interested in this technology. When do you plan to file the patent? Thanks,

>Bill

>

>William E. Beschorner, M.D.
>President and Chief Scientific Officer
>Ximerex, Inc.
>2614 N. 161 Ave.
>Omaha, NE 68116-2461
>Tel: (402)559-2235 Fax: (402)445-2535
>Website: www.ximerex.com

William E. Beschorner, M.D.
President and Chief Scientific Officer
Ximerex, Inc.
2614 N. 161 Ave.
Omaha, NE 68116-2461
Tel: (402)559-2235 Fax: (402)445-2535
Website: www.ximerex.com